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(54) Title: TUMOUR SUPPRESSOR GENE DBCCR1 AT 9q32-33

(57) Abstract

The application relates to the identification of a tumour suppressor locus at 9q32-33, and a gene within this region is disclosed. The gene is called "DBCCR1" for Deleted in Bladder Cancer Chromosome Region candidate 1. The application further relates to uses of these findings, in particular in the diagnostic, prophylactic and therapeutic treatment of cancer, especially bladder cancer. In particular, some forms of cancer are linked to hypermethylation based silencing of the DBCCR1 promoter or gene.

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Tumour Suppressor Gene DBCCR1 at 9q32-33

Field of the Invention

The present invention concerns materials and methods relating to the identification of a tumour suppressor locus at 9q32-33, and a gene within this region. The present invention further relates to applications of these findings, in particular in the diagnostic, prophylactic and therapeutic treatment of cancer, especially bladder cancer.

Background of the Invention

Molecular genetic and cytogenetic analyses have shown that multiple genetic alterations are involved in the genesis and progression of transitional cell carcinomas (TCCs), which are the most common form (approximately 90%) of malignant epithelial tumour of the bladder and upper urinary tract. Among these alterations, the loss of heterozygosity (LOH) or the deletion of chromosome 9q and/or 9p is the most frequent genetic alteration (>50%) in both superficial papillary and invasive TCC (Smeets et al., 1987; Olumi et al., 1990; Dalbagni et al., 1993; Habuchi et al., 1993; Knowles et al., 1994). LOH studies have demonstrated the frequent occurrence of LOH at all loci on both arms of chromosome 9 and cytogenetic studies have identified frequent monosomy 9 in TCC (Smeets et al., 1987; Vanni et al., 1988). Deletion mapping studies using microsatellite markers have defined localised deletions on the short arm and long arm of chromosome 9 (Ruppert et al., 1993; Keen and Knowles, 1994).

On 9p, candidate tumour suppressor genes, p16/CDKN2/MTS1 and p15/MTS2 identified at 9p21 have been found to be homozygously deleted in many types of human malignant tumours including TCC (Cairns et al., 1995; Williamson et al., 1995). On 9q, it has been shown that there are at least two common deleted regions, one at 9q13-31, and a second at 9q34, therefore indicating the presence of multiple tumour suppressor loci on 9q (Habuchi et al., 1995; Simoneau et al., 1996). The findings are supported by a cytogenetic study showing the localised deletion of

proximal 9q (Bernués et al., 1993). Furthermore, a microcell fusion study transferring normal human chromosome 9 into bladder cancer cells also indicates the presence of more than one tumour suppressor loca (Wu et al., 1996). The commonly deleted regions on 9q34 and 9q13-31 which have been defined by LOH analyses to date are relatively large (Habuchi et al., 1995; Simoneau et al., 1996).

Summary of the Invention

The present invention concerns the localisation of a common deleted region at 9q32-33 between D9S1848 and AFMA239XA9 by a detailed deletion mapping analysis using a large number of microsatellite markers and the identification of a tumour suppressor gene IB3089A located within a single 840kb YAC covering this region. The tumour suppressor locus at 9q32-33 and IB3089A gene are also referred to herein as "DBCCR1" for Deleted in Bladder Cancer Chromosome Region candidate 1. The gene was previously referred to as "DBC1" for "deleted in bladder cancer one" and the two terms are used herein interchangeably.

Furthermore, LOH on 9q or partial LOH involving 9q32-33 has been reported in other types of sporadic human malignant tumour such as squamous carcinoma, non-melanoma skin cancer, renal cell carcinoma, squamous cell carcinoma of the oesophagus and ovarian cancer (Ah-See et al., 1994; Quinn et al., 1994; Cairns et al., 1995; Miura et al., 1995; Devlin et al., 1996), implicating the possible inactivation of a tumour suppressor at this locus in many human cancers.

Although the gene was expressed in multiple human tissues including normal urothelium, lack of expression of the gene was found in several bladder cancer cells. Methylation analysis of a CpG island at its 5' end and de novo expression by a demethylating agent in bladder cancer cells indicate the involvement of hypermethylation-based silencing of the gene. While not wishing to be bound by

any particular explanation, it appears that the role of the DBCCR1 gene in the development of sporadic cancer, e.g. bladder cancer, involves either or both of two somatic events, namely the deletion of one allele of the gene and the hypermethylation of the remaining allele leading to reduced expression of the gene.

Accordingly, in one aspect, the present invention provides an isolated nucleic acid molecule comprising the nucleotide of the DBCCR1 gene as set out in figure 6, or alleles thereof. The present invention also provides nucleic acid molecules including polymorphisms of the figure 6 nucleic acid sequence, for example, the silent polymorphisms selected from the group consisting of T1036C, C2044A and T2642C (see below). The present invention also includes the first characterisation of the genomic structure of the DBCCR1 gene see the intron/exon information in table 2, as well as the sequencing of the coding region shown in figure 6. The sequence of the DBCCR1 promoter region is set out in figure 11.

In a further aspect, the present invention provides an isolated nucleic acid molecule comprising a nucleotide sequence encoding a DBCCR1 polypeptide having the amino acid sequence set out in figure 6.

The present invention further includes an isolated nucleic acid molecule which has a nucleotide sequence encoding a polypeptide which is a variant, derivative or allele of the DBCCR1 polypeptide including the amino acid sequence set out in figure 6. The present invention also includes fragments of the DBCCR1 gene excluding the ESTs used in the identification of the gene or found in search after its location. Preferably, the fragments encode polypeptides retaining a biological property of full length DBCCR1 polypeptide, and have lengths greater than about 350 nucleotide, more preferably greater than 450 nucleotides, and still more preferably greater than 600 nucleotides in length.

In a further aspect, the present invention provides an

isolated nucleic acid molecule encoding a polypeptide having a 80% sequence identity to the DBCCR1 polypeptide including the amino acid sequence set out in figure 6.

In a further aspect, the present invention provides an expression vector comprising any one of the above nucleic acid molecules operably linked to control sequences to direct its expression. In a further aspect, the present invention provides host cells transformed with the above vectors. As discussed below, vectors (e.g. viral vectors) comprising the DBCCR1 nucleic acid can be used in methods of gene therapy, e.g. to produce active DBCCR1 polypeptide in a population of cells.

In a further aspect, the present invention provides a method of producing a DBCCR1 polypeptide comprising culturing the above host cells and recovering the DBCCR1 polypeptide thus produced. Preferably, the method further comprises the step of recovering the DBCCR1 polypeptide.

In a further aspect, the present invention provides any of the above mentioned nucleic acid molecules for use in a method of medical treatment.

In a further aspect, the present invention provides a substance which is a DBCCR1 polypeptide encoded by the any one of the above nucleic acid molecules.

In one embodiment, the present invention provides a substance which is a DBCCR1 polypeptide including the amino acid sequence set out in figure 6. The present invention also includes substances which are polypeptides and have sequence identity, for example greater than 80% amino acid identity, to the DBCCR1 polypeptide including the amino acid sequence set out in figure 6. The present invention further includes polypeptides which are variants, derivatives or alleles of the DBCCR1 polypeptide having the sequence set out in figure 6.

In a further aspect, the present invention includes a substance which is a fragment or active portion or functional mimetic of a DBCCR1 polypeptide including the amino acid sequence of figure 6.

In a further aspect, the present invention provides the above substances for use in a method of medical treatment.

Preferably, the DBCCR1 polypeptides and nucleic acid molecules are used in the treatment of cancer, bladder cancer, squamous carcinoma, skin cancer, renal cell carcinoma, oesophageal cancer and ovarian cancer.

In a further aspect, the present invention provides antibodies capable of specifically binding to any one of the above DBCCR1 nucleic acid molecules or DBCCR1 polypeptides. Antibodies capable of specifically binding DBCCR1 polypeptides are especially useful as the DBCCR1 polypeptides do not have significant homology with other known proteins, and the antibodies can be used in their detection and/or characterisation.

In further aspects, the present invention provides pharmaceutical compositions comprising one or more of the above nucleic acid molecules, substances or antibodies. Such compositions will typically also include a pharmaceutically acceptable carrier.

In a further aspect, the present invention provides the DBCCR1 polypeptides set out above for use in a method of medical treatment.

In a further aspect, the present invention provides the use of the above substances in the preparation of a medicament for treating cancer, especially bladder cancer.

In a further aspect, the present invention provides the use of a DBCCR1 polypeptide as defined above as a molecular weight marker, e.g. as a standard on a gel (SDS-PAGE etc).

In a further aspect, the present invention provides the use of a polymorphism in the DBCCR1 nucleic acid sequence as a genetic marker within the 9q32-33 locus, and more particularly within the interval between D9S1848 and AFMA239XA9. The use of genetic markers is well known in the art and they are valuable tools for the identification and mapping of genes. Examples of polymorphisms include

the T1036C, C2044A and T2642C polymorphisms described below. By way of example, in this aspect of the invention, nucleic acid including one of the genetic markers can be detected in a test sample using a nucleic acid probe capable of hybridising to the nucleic acid sequence including one or more of the polymorphisms, or a complementary sequence thereof, by exposing the test nucleic acid to the probe under hybridising conditions and observing whether hybridisation takes place. Of course, this approach is generally applicable and can also be used to detect the presence of DBCCR1 nucleic acid, by employing a probe based on the DBCCR1 nucleic acid sequence shown in figure 6 or a complementary sequence thereof.

In a further aspect, the present invention provides a method for determining inactivation of the DBCCR1 gene in a biological sample from a patient, the method comprising:

(a) determining the level of expression of the polypeptide encoded by the DBCCR1 gene and having the amino acid sequence set out in figure 6 in a sample from a patient; and/or;

(b) determining whether the promoter region or 5' end of the DBCCR1 gene is hypermethylated in a sample from patient; and/or,

(c) determining whether all or a part of at least one of the DBCCR1 alleles is deleted in a nucleic acid sample from a patient;

wherein inactivation of the DBCCR1 gene indicates the presence of a tumour or a predisposition of the patient to cancer.

In a preferred aspect, the present invention provides a method for determining the inactivation of the DBCCR1 gene, the method comprising determining the methylation in the DBCCR1 promoter region or gene. Methods for quantitating methylation differences between nucleic acid sequences (i.e. determining whether a patient has an inactive DBCCR1 gene) are discussed below. These include:

(a) the use of methylation sensitive single

nucleotide primer extension (Ms-SNuPE).

(b) digestion of genomic DNA with methylation sensitive restriction enzymes followed by Southern analysis.

(c) PCR-based methylation assays utilizing(digestion of genomic DNA with methylation-sensitive restriction enzymes prior to PCR amplification.

The above methods can be carried out following the digestion of bisulphite-converted DNA.

The above determinations may be useful in assisting a physician in the diagnosis or prognosis of cancer, e.g the forms of cancer mentioned above that are associated with deletions in the 9q32-33 region. In a preferred embodiment, the method is used in the diagnosis or prognosis of bladder cancer. Methods for carrying out the above determinations are well known in the art and are discussed below.

In a further aspect, the present invention provides a method for detecting mutations in the DBCCR1 gene or the polypeptide encoded by the gene, the methods including:

(a) comparing the sequence of nucleic acid in the sample with the DBCCR1 nucleic acid sequence to determine whether the sample from the patient contains mutations; or,

(b) determining the presence in a sample from a patient of the polypeptide encoded by the DBCCR1 gene and, if present, determining whether the polypeptide is full length, and/or is mutated, and/or is expressed at the normal level; or,

(c) using DNA fingerprinting to compare the restriction pattern produced when a restriction enzyme cuts a sample of nucleic acid from the patient with the restriction pattern obtained from normal DBCCR1 gene or from known mutations thereof; or,

(d) using a specific binding member capable of binding to a DBCCR1 nucleic acid sequence (either a normal sequence or a known mutated sequence), the specific binding member comprising nucleic acid hybridisable with the DBCCR1

sequence, or substances comprising an antibody domain with specificity for a native or mutated DBCCR1 nucleic acid sequence or the polypeptide encoded by it, the specific binding member being labelled so that binding of the specific binding member to its binding partner is detectable; or,

(e) using PCR involving one or more primers based on normal or mutated DBCCR1 gene sequence to screen for normal or mutant DBCCR1 gene in a sample from a patient.

The above method includes detecting mutations in coding and non-coding sequences (exons/introns) of the DBCCR1 gene, and covers mutations which result in a change in the amino acid sequence of the DBCCR1 gene and mutations which are silent.

In a further aspect, the present invention provides the use of a methylation inhibitor in the preparation of a medicament for treating cancer, especially bladder cancer, wherein the methylation inhibitor causes activation of the tumour suppressor activity of the DBCCR1 gene, e.g. by allowing the production of active DBCCR1 polypeptide. An example of a methylation inhibitor is methylation inhibitor 5-aza-2'-deoxycytidine. The role of methylation in carcinogenesis and further examples of methylation inhibitors is discussed in Zingg and Jones, 1997.

In a further aspect, the present invention provides the use of the above DBCCR1 nucleic acid in designing primers for use in the polymerase chain reaction to amplify all or part of the DBCCR1 nucleic acid sequence. Examples of primers used to amplify the coding region of the DBCCR1 gene are set out in table 3.

Brief Description of the Figures

Aspects of the present invention will now be further described with reference to the accompanying figures by way of example and not limitation. Further aspects of the invention will be apparent to those of ordinary skill in the art.

Figure 1:

Localised deletion at 9q32-33 found in 5 TCCs. The 5 tumours depicted showed retention of heterozygosity at all other informative loci on 9q and 9p. Other examined loci are described in the Materials and Methods section. The order of markers from D9S103 to D9S195 and D9S258 has not been defined clearly. D9S275 has been mapped 1 cM proximal to D9S195 and D9S258 by Généthon.

Figure 2:

Representative autoradiographs showing the pattern of localised deletion at 9q32-33. N=normal DNA. T=tumour DNA. Deleted alleles are indicated with arrowheads in panels A, B and C. (A) In tumour #35, LOH is found at D9S195 with retention of heterozygosity at the other markers. (B) In tumour #68, LOH is observed at D9S195 and AFMA239ZE1 with retention of heterozygosity at the other markers. (C) In tumour #121, localised LOH is found at D9S195 with retention of heterozygosity at D9S1848, AFMA239ZE1 and AFMA239XA9. (D) Representative patterns for microsatellite markers D9S195, AFMA239ZE1 and D9S1848. In D9S195, stutter (ghost) bands were consistently observed both above and below the major band representing each allele. With this marker, a significant difference in intensity between each allele is observed normally (case #51,#84, #93 and #35 in A) where there is large difference in allele size. Cases #131 and #135 are constitutionally homozygous (not informative) at this locus and all other tumours shown have LOH. The markers AFMA239ZE1 and D9S1848 show no stutter bands above the major band. Tumour #23 shows loss of an upper allele and tumours #29 and #30 show clear loss of a lower allele at AFMA239ZE1. Note that heterozygotes for AFMA239ZE1 show significant difference in intensity between each allele differing only by one CA repeat unit as seen in case #68 (B). At D9S1848, tumours #43, #44, #53, #65, and #128 show retention of heterozygosity and all other cases are constitutionally homozygous.

Figure 3:

A YAC contig map encompassing the deleted region at 9q32-33. 3EG8, 21GH3, 36GD9, 15HD3, 12IB1, 28BB3, 9EE5, 9DC8 and 21GH3 are from the ICI YAC library and other YACs are from the CEPH YAC library. Black squares and white squares indicate presence or absence of a particular STS in each YAC, respectively. Hatched squares indicate the YAC-end STSs. -R and -L indicate right and left YAC-ends, respectively. The size of each YAC was determined by CHEF-gel electrophoresis, followed by Southern hybridisation with total human DNA. The size of all CEPH YACs shown was consistent with CEPH data.

Figure 4:

Further deletion mapping in the critical deleted region in 5 tumours with localised deletion at 9q32-33. The order of microsatellite markers was determined from the YAC contig map constructed. White squares, black squares, and hatched squares indicate retention of heterozygosity, LOH, and constitutional homozygote (not informative), respectively.

Figure 5:

Schematic view of the deleted region at 9q32-33 in TCC based on a YAC contig map. By LOH analysis, the common deleted region is localized between AFMA239XA9 and D9S1848. -L and -R indicate sequence tagged sites (STSs) derived from the left and right arm end of each YAC (Habuchi et al., 1997). EST IB3089 was placed between 9DC8-R and 814c5-L. 5′ end of the cDNA probe of the IB3089A cDNA hybridized with YACs 852e11, 15HD3 and 9DC8. However, the precise location of the 5′ end of IB3089A has not been defined. All YAC clones were from the CEPH or the ICI.

Figure 6:

cDNA sequence, predicted amino acid sequence and exon-intron boundaries of IB3089A. The proposed termination site, polyadenylation signals (Sheets et al., 1990; Wahle and Keller, 1992) and in-frame stop codons before the initiation codon are underlined. Each exon boundary is shown by >><<. The first 10 nucleotide sequence was obtained

by 5' RACE experiment. The last 4 nucleotides of sequence were from clones IB1708 and 28122. The remaining sequence was obtained from a clone ICRFp507K12270.

Figure 7:

IB3089A mRNA expression in adult tissues. Northern blots of 2 μg of poly(A)+ RNA from various adult tissues (Clontech) were probed with the insert of a clone ICRFp507K12270 (A) and a β-actin probe (B). The filter was exposed for 10 days at -70°C with intensifying screen. The main band in brain can be seen after 48 hour exposure. The filters were reprobed with the β-actin probe (Clontech). sk. muscle = skeletal muscle. blood = peripheral blood.

Figure 8:

RT-PCR analysis of IB3089A mRNA expression in bladder cancer cell lines. RT-PCR products were electrophoresed and blotted onto nylon membranes. Blots were hybridized with ^{32}P-labelled internal specific primers exposed for 0.5 to 2 hours. No bands were identified in cell lines 609CR, 5637, RT4, T24 and SCaBer even after a longer exposure. GAPDH RT-PCR was used as a control for RNA integrity and reverse transcription reaction.

Figure 9:

Methylation status of the IB3089A CpG island BssHII sites. (A) Brief map of the region surrounding exon 1, the probe used and sites for predicted BamHI and BssHII digestion. (B) DNA from normal tissues, bladder cancer cell lines, and primary TCCs of the bladder was digested with BamHI alone (-), or codigested with BamHI and BssHII (+). PL=normal placental tissue, PB=peripheral blood, T=primary TCCs of the bladder. The band size of DNA markers is indicated in kilobases.

Figure 10:

Induction of IB3089A mRNA expression in bladder cancer cell lines by a demethylating agent. Three cell lines (T24, 609CR and 5637), which do not express IB3089A mRNA, were treated with 5-aza-2'-deoxycytidine at a final concentration of 1 μM for 4 days. Controls were mock

treated with media. GAPDH RT-PCR was used as a control for RNA integrity and reverse transcription reaction.

Figure 11:

This figure depicts the nucleic acid sequence of the region containing the DBCCR1 promoter, the sequence starting immediately upstream of the the nucleic acid sequence shown in figure 6.

Detailed Description

Preparation of DBCCR1 nucleic acid, and vectors and host cells incorporating the nucleic acid.

The DBCCR1 coding sequence may be that shown in figure 6, or it may be a variant, derivative, or allele of this sequence, or the complement of any of these sequences. The sequence may differ from that shown by a change which is one or more of addition, insertion, deletion and substitution of one or more nucleotides of the sequence shown. Changes to a nucleotide sequence may result in an amino acid change at the protein level, or not, as determined by the genetic code.

Thus, nucleic acid according to the present invention may include a sequence different from the sequence shown in figure 6 yet encode a polypeptide with the same amino acid sequence. The amino acid sequence of the complete DBCCR1 polypeptide shown in figure 6 consists of 761 residues. The DBCCR1 promoter region is set out in figure 11.

On the other hand, the encoded polypeptide may comprise an amino acid sequence which differs by one or more amino acid residues from the amino acid sequence shown in figure 6. Nucleic acid encoding a polypeptide which is an amino acid sequence variant, derivative, or allele of the sequence shown in figure 6 is further provided by the present invention. Such polypeptides are discussed below. Nucleic acid encoding such a polypeptide may show greater than about 60% homology with the coding sequence shown in figure 6 greater than about 70% homology, greater than about 80% homology, greater than about 90% homology, or

greater than about 95% homology.

Generally, nucleic acid according to the present invention is provided as an isolate, in isolated and/or purified form, or free or substantially free of material with which it is naturally associated, such as free or substantially free of nucleic acid flanking the gene in the human genome, except possibly one or more regulatory sequence(s) for expression. Nucleic acid may be wholly or partially synthetic and may include genomic DNA, cDNA or RNA. Where nucleic acid according to the invention includes RNA, reference to the sequence shown should be construed as reference to the RNA equivalent, with U substituted for T.

Nucleic acid sequences encoding all or part of the DBCCR1 gene and/or its regulatory elements can be readily prepared by the skilled person using the information and references contained herein and techniques known in the art (for example, see Sambrook, Fritsch and Maniatis, "Molecular Cloning, A Laboratory Manual", Cold Spring Harbor Laboratory Press, 1989, and Ausubel et al, "Short Protocols in Molecular Biology", John Wiley and Sons, 1992). These techniques include (i) the use of the polymerase chain reaction (PCR) to amplify samples of such nucleic acid, e.g. from genomic sources, (ii) chemical synthesis, or (iii) preparing cDNA sequences. Modifications to the DBCCR1 sequences can be made, e.g. using site directed mutagenesis, to lead to the expression of modified DBCCR1 polypeptide or to take account of codon preference in the host cells used to express the nucleic acid.

In order to obtain expression of the DBCCR1 nucleic acid sequences, DBCCR1 sequences can be incorporated in a vector having control sequences operably linked to the DBCCR1 nucleic acid to control its expression. The vectors may include other sequences such as promoters or enhancers to drive the expression of the inserted nucleic acid, nucleic acid sequences so that the DBCCR1 polypeptide is

produced as a fusion and/or nucleic acid encoding secretion signals so that the polypeptide produced in the host cell is secreted from the cell. DBCCR1 polypeptide can then be obtained by transforming the vectors into host cells in which the vector is functional, culturing the host cells so that the DBCCR1 polypeptide is produced and recovering the DBCCR1 polypeptide from the host cells or the surrounding medium. Prokaryotic and eukaryotic cells are used for this purpose in the art, including strains of E. coli, yeast, and eukaryotic cells such as COS or CHO cells. The choice of host cell can be used to control the properties of the DBCCR1 polypeptide expressed in those cells, e.g. controlling where the polypeptide is deposited in the host cells or affecting properties such as its glycosylation.

PCR techniques for the amplification of nucleic acid are described in US Patent No. 4,683,195. In general, such techniques require that sequence information from the ends of the target sequence is known to allow suitable forward and reverse oligonucleotide primers to be designed to be identical or similar to the polynucleotide sequence that is the target for the amplification. PCR comprises steps of denaturation of template nucleic acid (if double-stranded), annealing of primer to target, and polymerisation. The nucleic acid probed or used as template in the amplification reaction may be genomic DNA, cDNA or RNA. PCR can be used to amplify specific sequences from genomic DNA, specific RNA sequences and cDNA transcribed from mRNA, bacteriophage or plasmid sequences. The DBCCR1 nucleic acid sequences provided herein readily allow the skilled person to design PCR primers, see for example table 2. References for the general use of PCR techniques include Mullis et al, Cold Spring Harbor Symp. Quant. Biol., 51:263, (1987), Ehrlich (ed), PCR technology, Stockton Press, NY, 1989, Ehrlich et al, Science, 252:1643-1650, (1991), "PCR protocols; A Guide to Methods and Applications", Eds. Innis et al, Academic Press, New York, (1990).

The nucleic acid sequences provided in figures 6 or 11 are useful for identifying nucleic acid of interest (and which may be according to the present invention) in a test sample. The present invention provides a method of obtaining nucleic acid of interest, the method including hybridisation of a probe having the sequence shown in figures 6 or 11, or a complementary sequence, to target nucleic acid. This approach can be extended to polymorphisms in the figure 6 sequence which can, for example, be used as markers in the 9q32-33 region of the genome.

Hybridisation is generally followed by identification of successful hybridisation and isolation of nucleic acid which has hybridised to the probe, which may involve one or more steps of PCR.

Nucleic acid according to the present invention is obtainable using one or more oligonucleotide probes or primers designed to hybridise with one or more fragments of the nucleic acid sequence shown in figure 6 or 11, particularly fragments of relatively rare sequence, based on codon usage or statistical analysis. A primer designed to hybridise with a fragment of the nucleic acid sequence shown in the above figures may be used in conjunction with one or more oligonucleotides designed to hybridise to a sequence in a cloning vector within which target nucleic acid has been cloned, or in so-called "RACE" (rapid amplification of cDNA ends) in which cDNA's in a library are ligated to an oligonucleotide linker and PCR is performed using a primer which hybridises with the sequence shown in figures 6 or 11 and a primer which hybridises to the oligonucleotide linker.

Such oligonucleotide probes or primers, as well as the full-length sequence (and alleles, mutants, variants and derivatives) are also useful in screening a test sample containing nucleic acid for the presence of polymorphisms, the probes hybridising with a target sequence from a sample obtained from the individual being tested. The conditions

of the hybridisation can be controlled to minimise non-specific binding, and preferably stringent to moderately stringent hybridisation conditions are preferred. The skilled person is readily able to design such probes, label them and devise suitable conditions for the hybridisation reactions, assisted by textbooks such as Sambrook et al (1989) and Ausubel et al (1992).

As well as determining the presence of polymorphisms or mutations in the DBCCR1 sequence, the probes may also be used to determine whether mRNA encoding DBCCR1 is present in a cell or tissue.

The finding that CpG islands at the 5' end of the DBCCR1 gene and in the promoter region are hypermethylated in some types of cancer means that samples of DBCCR1 nucleic acid including this part of the DBCCR1 gene or promoter region can be screened to determine the extent of hypermethylation, and thereby to assist in the prognosis or diagnosis of cancers, such as bladder cancer, in a patient. Protocols for determining the extent of hypermethylation are described below.

Nucleic acid isolated and/or purified from one or more cells (e.g. human) or a nucleic acid library derived from nucleic acid isolated and/or purified from cells (e.g. a cDNA library derived from mRNA isolated from the cells), may be probed under conditions for selective hybridisation and/or subjected to a specific nucleic acid amplification reaction such as the polymerase chain reaction (PCR).

In the context of cloning, it may be necessary for one or more gene fragments to be ligated to generate a full-length coding sequence. Also, where a full-length encoding nucleic acid molecule has not been obtained, a smaller molecule representing part of the full molecule, may be used to obtain full-length clones. Inserts may be prepared from partial cDNA clones and used to screen cDNA libraries. The full-length clones isolated may be subcloned into expression vectors and activity assayed by transfection into suitable host cells, e.g. with a reporter plasmid.

A method may include hybridisation of one or more (e.g. two) probes or primers to target nucleic acid. Where the nucleic acid is double-stranded DNA, hybridisation will generally be preceded by denaturation to produce single-stranded DNA. The hybridisation may be as part of a PCR procedure, or as part of a probing procedure not involving PCR. An example procedure would be a combination of PCR and low stringency hybridisation. A screening procedure, chosen from the many available to those skilled in the art, is used to identify successful hybridisation events and isolated hybridised nucleic acid.

Binding of a probe to target nucleic acid (e.g. DNA) may be measured using any of a variety of techniques at the disposal of those skilled in the art. For instance, probes may be radioactively, fluorescently or enzymatically labelled. Other methods not employing labelling of probe include examination of restriction fragment length polymorphisms, amplification using PCR, RNAase cleavage and allele specific oligonucleotide probing.

Probing may employ the standard Southern blotting technique. For instance DNA may be extracted from cells and digested with different restriction enzymes. Restriction fragments may then be separated by electrophoresis on an agarose gel, before denaturation and transfer to a nitrocellulose filter. Labelled probe may be hybridised to the DNA fragments on the filter and binding determined. DNA for probing may be prepared from RNA preparations from cells.

Preliminary experiments may be performed by hybridising under low stringency conditions various probes to Southern blots of DNA digested with restriction enzymes. Suitable conditions would be achieved when a large number of hybridising fragments were obtained while the background hybridisation was low. Using these conditions nucleic acid libraries, e.g. cDNA libraries representative of expressed sequences, may be searched.

Those skilled in the art are well able to employ

suitable conditions of the desired stringency for selective hybridisation, taking into account factors such as oligonucleotide length and base composition, temperature and so on.

On the basis of amino acid sequence information, oligonucleotide probes or primers may be designed, taking into account the degeneracy of the genetic code, and, where appropriate, codon usage of the organism from the candidate nucleic acid is derived. An oligonucleotide for use in nucleic acid amplification may have about 10 or fewer codons (e.g. 6, 7 or 8), i.e. be about 30 or fewer nucleotides in length (e.g. 18, 21 or 24). Generally specific primers are upwards of 14 nucleotides in length, but not more than 18-20. Those skilled in the art are well versed in the design of primers for use processes such as PCR.

A further aspect of the present invention provides an oligonucleotide or polynucleotide fragment of the nucleotide sequence shown in figures 6 or 11, or a complementary sequence, in particular for use in a method of obtaining and/or screening nucleic acid. The sequences referred to above may be modified by addition, substitution, insertion or deletion of one or more nucleotides, but preferably without abolition of ability to hybridise selectively with nucleic acid with the sequence shown in figure 6, that is wherein the degree of homology of the oligonucleotide or polynucleotide with one of the sequences given is sufficiently high.

Nucleic acid according to the present invention may be used in methods of gene therapy, for instance in treatment of individuals with the aim of preventing or curing (wholly or partially) cancer. This too is discussed below.

Nucleic acid according to the present invention, such as a full-length coding sequence or oligonucleotide probe or primer, may be provided as part of a kit, e.g. in a suitable container such as a vial in which the contents are protected from the external environment. The kit may

include instructions for use of the nucleic acid, e.g. in PCR and/or a method for determining the presence of nucleic acid of interest in a test sample. A kit wherein the nucleic acid is intended for use in PCR may include one or more other reagents required for the reaction, such as polymerase, nucleosides, buffer solution etc. The nucleic acid may be labelled. A kit for use in determining the presence or absence of nucleic acid of interest may include one or more articles and/or reagents for performance of the method, such as means for providing the test sample itself, e.g. a swab for removing cells from the buccal cavity or a syringe for removing a blood sample or a container for collecting urine samples (such components generally being sterile).

A convenient way of producing a polypeptide according to the present invention is to express nucleic acid encoding it, by use of the nucleic acid in an expression system. The use of expression system has reached an advanced degree of sophistication today.

Accordingly, the present invention also encompasses a method of making a polypeptide (as disclosed), the method including expression from nucleic acid encoding the polypeptide (generally nucleic acid according to the invention). This may conveniently be achieved by growing a host cell in culture, containing such a vector, under appropriate conditions which cause or allow expression of the polypeptide. Polypeptides may also be expressed in in vitro systems, such as reticulocyte lysate.

Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. Suitable host cells include bacteria, eukaryotic cells such as mammalian and yeast, and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells, COS cells and many others. A common, preferred bacterial host is E. coli.

Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. 'phage, or phagemid, as appropriate. For further details see, for example, Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in Current Protocols in Molecular Biology, Ausubel et al. eds., John Wiley & Sons, 1992.

Thus, a further aspect of the present invention provides a host cell containing nucleic acid as disclosed herein. The nucleic acid of the invention may be integrated into the genome (e.g. chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard techniques. The nucleic acid may be on an extra-chromosomal vector within the cell.

A still further aspect provides a method which includes introducing the nucleic acid into a host cell. The introduction, which may (particularly for *in vitro* introduction) be generally referred to without limitation as "transformation", may employ any available technique. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g. vaccinia or, for insect cells, baculovirus. For bacterial cells, suitable techniques may include calcium chloride transformation, electroporation and transfection using bacteriophage. As an alternative, direct injection of the nucleic acid could be employed.

Marker genes such as antibiotic resistance or sensitivity genes may be used in identifying clones containing nucleic acid of interest, as is well known in the art.

The introduction may be followed by causing or allowing expression from the nucleic acid, e.g. by culturing host cells (which may include cells actually transformed although more likely the cells will be descendants of the transformed cells) under conditions for expression of the gene, so that the encoded polypeptide is produced. If the polypeptide is expressed coupled to an appropriate signal leader peptide it may be secreted from the cell into the culture medium. Following production by expression, a polypeptide may be isolated and/or purified from the host cell and/or culture medium, as the case may be, and subsequently used as desired, e.g. in the formulation of a composition which may include one or more additional components, such as a pharmaceutical composition which includes one or more pharmaceutically acceptable excipients, vehicles or carriers (e.g. see below).

Introduction of nucleic acid may take place in vivo by way of gene therapy, as discussed below.

A host cell containing nucleic acid according to the present invention, e.g. as a result of introduction of the nucleic acid into the cell or into an ancestor of the cell and/or genetic alteration of the sequence endogenous to the cell or ancestor (which introduction or alteration may take place in vivo or ex vivo), may be comprised (e.g. in the soma) within an organism which is an animal, particularly a mammal, which may be human or non-human, such as rabbit, guinea pig, rat, mouse or other rodent, cat, dog, pig, sheep, goat, cattle or horse, or which is a bird, such as a chicken. Genetically modified or transgenic animals or birds comprising such a cell are also provided as further aspects of the present invention, including knockout animals in which all or part of the DBCCR1 gene is deleted or non-functional.

This may have a therapeutic aim. (Gene therapy is discussed below.) The presence of a allele or variant sequence within cells of an organism, particularly when in place of a homologous endogenous sequence, may allow the organism to be used as a model in testing and/or studying the role of the DBCCR1 gene or substances which modulate activity of the encoded polypeptide in vitro or the promoter sequence shown in figure 11 are otherwise indicated to be of therapeutic potential.

Instead of or as well as being used for the production of a polypeptide encoded by a transgene, host cells may be used as a nucleic acid factory to replicate the nucleic acid of interest in order to generate large amounts of it. Multiple copies of nucleic acid of interest may be made within a cell when coupled to an amplifiable gene such as DHFR. Host cells transformed with nucleic acid of interest, or which are descended from host cells into which nucleic acid was introduced, may be cultured under suitable conditions, e.g. in a fermenter, taken from the culture and subjected to processing to purify the nucleic acid. Following purification, the nucleic acid or one or more fragments thereof may be used as desired, for instance in a diagnostic or prognostic assay as discussed elsewhere herein.

Production of DBCCR1 Polypeptides.

The skilled person can use the techniques described herein and others well known in the art to produce large amounts of the DBCCR1 polypeptide, or fragments or active portions thereof, for use as pharmaceuticals, in the developments of drugs and for further study into its properties and role in vivo.

Thus, a further aspect of the present invention provides a polypeptide which has the amino acid sequence shown in figure 6, which may be in isolated and/or purified form, free or substantially free of material with which it is naturally associated, such as other polypeptides or such

as human polypeptides other than DBCCR1 polypeptide or (for example if produced by expression in a prokaryotic cell) lacking in native glycosylation, e.g. unglycosylated.

Polypeptides which are amino acid sequence variants, alleles or derivatives are also provided by the present invention. A polypeptide which is a variant, allele, or derivative may have an amino acid sequence which differs from that given in figure 6 by one or more of addition, substitution, deletion and insertion of one or more amino acids. Preferred such polypeptides have DBCCR1 function, that is to say have one or more of the following properties: immunological cross-reactivity with an antibody reactive the polypeptide for which the sequence is given in figure 6; sharing an epitope with the polypeptide for which the amino acid sequence is shown in figure 6 (as determined for example by immunological cross-reactivity between the two polypeptides); having the tumour suppressing or growth inhibiting activity of full length DBCCR1. This latter activity can be assayed by transforming cells with a nucleic acid encoding the DBCCR1 polypeptide and observing any change in tumour suppression or growth inhibition takes place. Preferably, the cells do not express endogenous DBCCR1 polypeptide, e.g. the "5637" cell line used in the examples below), although with suitable controls cell types could be used.

A polypeptide which is an amino acid sequence variant, allele or derivative of the amino acid sequence shown in figure 6 may comprise an amino acid sequence which shares greater than about 35% sequence identity with the sequence shown, greater than about 40%, greater than about 50%, greater than about 60%, greater than about 70%, greater than about 80%, greater than about 90% or greater than about 95%. The sequence may share greater than about 60% similarity, greater than about 70% similarity, greater than about 80% similarity or greater than about 90% similarity with the amino acid sequence shown in any one of figure 6. Particular amino acid sequence variants may differ from

those shown in figure 6 by insertion, addition, substitution or deletion of 1 amino acid, 2, 3, 4, 5-10, 10-20 20-30, 30-50, 50-100, 100-150, or more than 150 amino acids. Sequence comparison can be made using the GCG program which is available from Genetics Computer Group, Oxford Molecular Group, Madison, Wisconsin, USA, Version 9.1.

The present invention also includes active portions, fragments, derivatives and functional mimetics of the DBCCR1 polypeptides of the invention.

An "active portion" of DBCCR1 polypeptide means a peptide which is less than said full length DBCCR1 polypeptide, but which retains its essential biological activity, i.e. inhibiting cell growth or acting as a tumour suppressor.

A "fragment" of the DBCCR1 polypeptide means a stretch of amino acid residues of at least about five to seven contiguous amino acids, often at least about seven to nine contiguous amino acids, typically at least about nine to 13 contiguous amino acids and, most preferably, at least about 20 to 30 or more contiguous amino acids. Fragments of the DBCCR1 polypeptide sequence antigenic determinants or epitopes useful for raising antibodies to a portion of the DBCCR1 amino acid sequence.

A "derivative" of the DBCCR1 polypeptide or a fragment thereof means a polypeptide modified by varying the amino acid sequence of the protein, e.g. by manipulation of the nucleic acid encoding the protein or by altering the protein itself. Such derivatives of the natural amino acid sequence may involve insertion, addition, deletion or substitution of 1 amino acid, 2, 3, 4, 5-10, 10-20 20-30, 30-50, 50-100, 100-150, or more than 150 amino acids, without fundamentally altering a given biological activity of the wild type DBCCR1 polypeptide.

"Functional mimetic" means a substance which may not contain an active portion of the DBCCR1 amino acid sequence, and probably is not a peptide at all, but which

retains the essential biological activity of natural DBCCR1 polypeptide. The design and screening of candidate mimetics is described in detail below.

A polypeptide according to the present invention may be isolated and/or purified (e.g. using an antibody) for instance after production by expression from encoding nucleic acid (for which see below). Polypeptides according to the present invention may also be generated wholly or partly by chemical synthesis. The isolated and/or purified polypeptide may be used in formulation of a composition, which may include at least one additional component, for example a pharmaceutical composition including a pharmaceutically acceptable excipient, vehicle or carrier. A composition including a polypeptide according to the invention may be used in prophylactic and/or therapeutic treatment as discussed below.

The DBCCR1 polypeptides can also be linked to a coupling partner, e.g. an effector molecule, a label, a drug, a toxin and/or a carrier or transport molecule. Techniques for coupling the peptides of the invention to both peptidyl and non-peptidyl coupling partners are well known in the art. In one embodiment, the carrier molecule is a 16 aa peptide sequence derived from the homeodomain of *Antennapedia* (e.g. as sold under the name "Penetratin"), which can be coupled to a peptide via a terminal Cys residue. The "Penetratin" molecule and its properties are described in WO 91/18981.

A polypeptide, peptide fragment, allele or variant according to the present invention may be used as an immunogen or otherwise in obtaining specific antibodies. Antibodies are useful in purification and other manipulation of polypeptides and peptides, diagnostic screening and therapeutic contexts. This is discussed further below.

A polypeptide according to the present invention may be used in screening for molecules which affect or modulate its activity or function. Such molecules may be useful in

a therapeutic (possibly including prophylactic) context.

Production of DBCCR1 Antibodies.

A further important use of the DBCCR1 polypeptides is in raising antibodies that have the property of specifically binding to the DBCCR1 polypeptides, or fragments or active portions thereof. As the DBCCR1 polypeptide of figure 6 does not have significant homology with known polypeptides, so antibodies raised against this polypeptide will be new.

The production of monoclonal antibodies is well established in the art. Monoclonal antibodies can be subjected to the techniques of recombinant DNA technology to produce other antibodies or chimeric molecules which retain the specificity of the original antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the complementarity determining regions (CDRs), of an antibody to the constant regions, or constant regions plus framework regions, of a different immunoglobulin. See, for instance, EP-A-184187, GB-A-2188638 or EP-A-239400. A hybridoma producing a monoclonal antibody may be subject to genetic mutation or other changes, which may or may not alter the binding specificity of antibodies produced.

The provision of the novel DBCCR1 polypeptides enables for the first time the production of antibodies able to bind it specifically. Accordingly, a further aspect of the present invention provides an antibody able to bind specifically to the polypeptide whose sequence is given in figure 6. Such an antibody may be specific in the sense of being able to distinguish between the polypeptide it is able to bind and other human polypeptides for which it has no or substantially no binding affinity (e.g. a binding affinity of about 1000x worse). Specific antibodies bind an epitope on the molecule which is either not present or is not accessible on other molecules. Antibodies according to the present invention may be specific for the wild-type

polypeptide. Antibodies according to the invention may be specific for a particular variant, allele, mutant or derivative polypeptide as between that molecule and the wild-type DBCCR1 polypeptide, so as to be useful in diagnostic and prognostic methods as discussed below. Antibodies are also useful in purifying the polypeptide or polypeptides to which they bind, e.g. following production by recombinant expression from encoding nucleic acid.

Preferred antibodies according to the invention are isolated, in the sense of being free from contaminants such as antibodies able to bind other polypeptides and/or free of serum components. Monoclonal antibodies are preferred for some purposes, though polyclonal antibodies are within the scope of the present invention.

Antibodies may be obtained using techniques which are standard in the art. Methods of producing antibodies include immunising a mammal (e.g. mouse, rat, rabbit, horse, goat, sheep or monkey) with the protein or a fragment thereof. Antibodies may be obtained from immunised animals using any of a variety of techniques known in the art, and screened, preferably using binding of antibody to antigen of interest. For instance, Western blotting techniques or immunoprecipitation may be used (Armitage et al, Nature, 357:80-82, 1992). Isolation of antibodies and/or antibody-producing cells from an animal may be accompanied by a step of sacrificing the animal.

As an alternative or supplement to immunising a mammal with a peptide, an antibody specific for a protein may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, e.g. using lambda bacteriophage or filamentous bacteriophage which display functional immunoglobulin binding domains on their surfaces; for instance see WO92/01047. The library may be naive, that is constructed from sequences obtained from an organism which has not been immunised with any of the proteins (or fragments), or may be one constructed using sequences obtained from an organism which has been exposed

to the antigen of interest.

Antibodies according to the present invention may be modified in a number of ways. Indeed the term "antibody" should be construed as covering any binding substance having a binding domain with the required specificity. Thus the invention covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including synthetic molecules and molecules whose shape mimics that of an antibody enabling it to bind an antigen or epitope.

Example antibody fragments, capable of binding an antigen or other binding partner are the Fab fragment consisting of the VL, VH, Cl and CH1 domains; the Fd fragment consisting of the VH and CH1 domains; the Fv fragment consisting of the VL and VH domains of a single arm of an antibody; the dAb fragment which consists of a VH domain; isolated CDR regions and F(ab')2 fragments, a bivalent fragment including two Fab fragments linked by a disulphide bridge at the hinge region. Single chain Fv fragments are also included.

Humanised antibodies in which CDRs from a non-human source are grafted onto human framework regions, typically with the alteration of some of the framework amino acid residues, to provide antibodies which are less immunogenic than the parent non-human antibodies, are also included within the present invention

A hybridoma producing a monoclonal antibody according to the present invention may be subject to genetic mutation or other changes. It will further be understood by those skilled in the art that a monoclonal antibody can be subjected to the techniques of recombinant DNA technology to produce other antibodies or chimeric molecules which retain the specificity of the original antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the complementarity determining regions (CDRs), of an antibody to the constant regions, or constant regions plus framework regions, of a

different immunoglobulin. See, for instance, EP-A-184187, GB-A-2188638 or EP-A-0239400. Cloning and expression of chimeric antibodies are described in EP-A-0120694 and EP-A-0125023.

Hybridomas capable of producing antibody with desired binding characteristics are within the scope of the present invention, as are host cells, eukaryotic or prokaryotic, containing nucleic acid encoding antibodies (including antibody fragments) and capable of their expression. The invention also provides methods of production of the antibodies including growing a cell capable of producing the antibody under conditions in which the antibody is produced, and preferably secreted.

The reactivities of antibodies on a sample may be determined by any appropriate means. Tagging with individual reporter molecules is one possibility. The reporter molecules may directly or indirectly generate detectable, and preferably measurable, signals. The linkage of reporter molecules may be directly or indirectly, covalently, e.g. via a peptide bond or non-covalently. Linkage via a peptide bond may be as a result of recombinant expression of a gene fusion encoding antibody and reporter molecule.

One favoured mode is by covalent linkage of each antibody with an individual fluorochrome, phosphor or laser dye with spectrally isolated absorption or emission characteristics. Suitable fluorochromes include fluorescein, rhodamine, phycoerythrin and Texas Red. Suitable chromogenic dyes include diaminobenzidine.

Other reporters include macromolecular colloidal particles or particulate material such as latex beads that are coloured, magnetic or paramagnetic, and biologically or chemically active agents that can directly or indirectly cause detectable signals to be visually observed, electronically detected or otherwise recorded. These molecules may be enzymes which catalyse reactions that develop or change colours or cause changes in electrical

properties, for example. They may be molecularly excitable, such that electronic transitions between energy states result in characteristic spectral absorptions or emissions. They may include chemical entities used in conjunction with biosensors. Biotin/avidin or biotin/streptavidin and alkaline phosphatase detection systems may be employed.

The mode of determining binding is not a feature of the present invention and those skilled in the art are able to choose a suitable mode according to their preference and general knowledge.

Antibodies according to the present invention may be used in screening for the presence of a polypeptide, for example in a test sample containing cells or cell lysate as discussed, and may be used in purifying and/or isolating a polypeptide according to the present invention, for instance following production of the polypeptide by expression from encoding nucleic acid therefor. Antibodies may modulate the activity of the polypeptide to which they bind and so, if that polypeptide has a deleterious effect in an individual, may be useful in a therapeutic context (which may include prophylaxis).

An antibody may be provided in a kit, which may include instructions for use of the antibody, e.g. in determining the presence of a particular substance in a test sample. One or more other reagents may be included, such as labelling molecules, buffer solutions, elutants and so on. Reagents may be provided within containers which protect them from the external environment, such as a sealed vial.

Diagnostic Methods.

A number of methods are known in the art for analysing biological samples from individuals to determine the extent of inactivation of the DBCCR1 gene in a biological sample from a patient. The purpose of such analysis may be used for diagnosis or prognosis, and serve to detect the

presence of an existing cancer, to help identify the type of cancer, to assist a physician in determining the severity or likely course of the cancer and/or to optimise treatment of it.

One aspect of the invention involves methods for:

(a) determining the level of expression of the polypeptide encoded by the DBCCR1 gene and having the amino acid sequence set out in figure 6 in a sample from a patient; and/or;

(b) determining whether the promoter or 5'end of the DBCCR1 gene is hypermethylated in a sample from patient; and/or,

(c) determining whether all or a part of at least one of the DBCCR1 alleles is deleted in a nucleic acid sample from a patient.

Examples of methods for carrying out (a) to (c) are given in the examples below, although the person skilled in the art will be aware of or readily able to devise alternative protocols. As regards (b), as some restriction enzymes that cut at CpG are prevented from doing so by the presence of methylation, nucleic acid samples including the 5' end of the DBCCR1 gene can be exposed to such enzymes and the resulting fragments analysed to determine the presence and/or amount of methylation at this site, visualising the fragments produced using well known methods such as Southern blotting or PCR and sizing the fragments on a gel.

Preferred methods for determining inactivation of the DBCCR1 gene by determining the methylation in the DBCCR1 promoter region or gene are generally described in Gonzalgo et al, Nucleic Acids Research, 25(12):2529-2531, 1997. Methods for quantitating methylation differences between nucleic acid sequences (i.e. determining whether a patient has an inactive DBCCR1 gene) are discussed below. These include:

(a) the use of methylation sensitive single nucleotide primer extension (Ms-SNuPE).

(b) digestion of genomic DNA with methylation sensitive restriction enzymes followed by Southern analysis.

(c) PCR-based methylation assays utilizing digestion of genomic DNA with methylation-sensitive restriction enzymes prior to PCR amplification (see Singer-Sam et al, Nucleic Acids Research, 18:687, 1990).

The above methods can carried out following the digestion of bisulphite-converted DNA to convert unmethylated cytosine to be converted to uracil. The use of bisulphite to treat the DNA means that the uracil from the unmethylated cytosine is replicated as thymine during subsequent PCR. However, methylcytosine is resistant to deamination and is therefore replicated as cytosine during amplification. Thus, quantitation of the ratio of methylated:unmethylated cytosine (C:T) allows the extent of methylation in a nucleic acid sequence to be determined and correlated to standards, e.g. to diagnose the presence of a tumour or a urothelial condition predisposed to develop cancer. By way of example, the C:T ratio can be determined by incubating the isolated PCR product, primers and Taq polymerase with either [^{32}P]dCTP or [^{32}P]dTTP followed by denaturing gel electrophoresis and phosphoimage analysis.

Alternatively, the PCR product may be subjected to restriction analysis to assess methylation at specific sites or in Ms-SNuPE by annealing specific primers which terminate immediately 5' of the nucleotide to be assessed, followed by primer extension in the presence of [^{32}P]dCTP or [^{32}P]dTTP, gel electrophoresis and phosphoimage analysis to determine the ratio of C:T incorporated.

The present invention also includes method for detecting DBCCR1 nucleic acid or polypeptides, in particular mutations in the DBCCR1 nucleic acid or polypeptides associated with cancer, the methods including:

(a) comparing the sequence of nucleic acid in the sample with the DBCCR1 nucleic acid sequence to determine whether the sample from the patient contains mutations; or,

(b) determining the presence in a sample from a patient of the polypeptide encoded by the DBCCR1 gene and, if present, determining whether the polypeptide is full length, and/or is mutated, and/or is expressed at the normal level; or,

(c) using DNA fingerprinting to compare the restriction pattern produced when a restriction enzyme cuts a sample of nucleic acid from the patient with the restriction pattern obtained from normal DBCCR1 gene or from known mutations thereof; or,

(d) using a specific binding member capable of binding to a DBCCR1 nucleic acid sequence (either a normal sequence or a known mutated sequence), the specific binding member comprising nucleic acid hybridisable with the DBCCR1 sequence, or substances comprising an antibody domain with specificity for a native or mutated DBCCR1 nucleic acid sequence or the polypeptide encoded by it, the specific binding member being labelled so that binding of the specific binding member to its binding partner is detectable; or,

(e) using PCR involving one or more primers based on normal or mutated DBCCR1 gene sequence to screen for normal or mutant DBCCR1 gene in a sample from a patient.

The above methods are equally applicable to detecting mutations in the coding or the non-coding sequence of the DBCCR1 gene, and to detecting silent mutations as well as mutations changing the amino acid sequence of the DBCCR1 polypeptide.

A "specific binding pair" comprises a specific binding member (sbm) and a binding partner (bp) which have a particular specificity for each other and which in normal conditions bind to each other in preference to other molecules. Examples of specific binding pairs are antigens and antibodies, molecules and receptors and complementary nucleotide sequences. The skilled person will be able to think of many other examples and they do not need to be listed here. Further, the term "specific binding pair" is

also applicable where either or both of the specific binding member and the binding partner comprise a part of a larger molecule. In embodiments in which the specific binding pair are nucleic acid sequences, they will be of a length to hybridise to each other under the conditions of the assay, preferably greater than 10 nucleotides long, more preferably greater than 15 or 20 nucleotides long.

The methods can be carried out using biological samples which include blood, plasma, serum, tissue samples, tumour samples, saliva and urine. The use of urine sample in methods for the diagnosis or prognosis of bladder cancer is especially convenient.

There are various methods for determining the presence or absence in a test sample of a particular nucleic acid sequence, such as the sequence shown in figures 6 or 11 or a mutant, variant or allele thereof. Exemplary tests include nucleotide sequencing, hybridization using nucleic acid immobilized on chips, molecular phenotype tests, protein truncation tests (PTT), single-strand conformation polymorphism (SSCP) tests, mismatch cleavage detection and denaturing gradient gel electrophoresis (DGGE). These techniques and their advantages and disadvantages are reviewed in Nature Biotechnology, 15:422-426, 1997. Homozygous deletion of all or part of the DBCCR1 gene can be assessed using Southern blotting or quantitative duplex PCR.

Tests may be carried out on preparations containing genomic DNA, cDNA and/or mRNA. Testing cDNA or mRNA has the advantage of the complexity of the nucleic acid being reduced by the absence of intron sequences, but the possible disadvantage of extra time and effort being required in making the preparations. RNA is more difficult to manipulate than DNA because of the wide-spread occurrence of RN'ases.

Nucleic acid in a test sample may be sequenced and the sequence compared with the sequence shown in figures 6 or 11, to determine whether or not a difference is present.

The nucleic acid may also be analysed to determine other differences between the normal gene and that found in cancer patients, e.g. by looking at the extent of methylation of CpG islands at the 5' end of the gene or in the promoter region shown in figure 11.

Since it will not generally be time- or labour-efficient to sequence all nucleic acid in a test sample or even the whole DBCCR1 gene, a specific amplification reaction such as PCR using one or more pairs of primers may be employed to amplify the region of interest in the nucleic acid, for instance the DBCCR1 gene or a particular region in which mutations associated with cancer. Exemplary primers for this purpose are shown in table 2. The amplified nucleic acid may then be sequenced as above, and/or tested in any other way to determine the presence or absence of a particular feature. Nucleic acid for testing may be prepared from nucleic acid removed from cells or in a library using a variety of other techniques such as restriction enzyme digest and electrophoresis.

Nucleic acid may be screened using a variant- or allele-specific probe. Such a probe corresponds in sequence to a region of the DBCCR1 gene, or its complement, containing a sequence alteration known to be associated with cancer. Under suitably stringent conditions, specific hybridisation of such a probe to test nucleic acid is indicative of the presence of the sequence alteration in the test nucleic acid. For efficient screening purposes, more than one probe may be used on the same test sample.

Allele- or variant-specific oligonucleotides may similarly be used in PCR to specifically amplify particular sequences if present in a test sample. Assessment of whether a PCR band contains a gene variant may be carried out in a number of ways familiar to those skilled in the art. The PCR product may for instance be treated in a way that enables one to display the mutation or polymorphism on a denaturing polyacrylamide DNA sequencing gel, with specific bands that are linked to the gene variants being

selected.

An alternative or supplement to looking for the presence of variant sequences in a test sample is to look for the presence of the normal sequence, e.g. using a suitably specific oligonucleotide probe or primer.

Approaches which rely on hybridisation between a probe and test nucleic acid and subsequent detection of a mismatch may be employed. Under appropriate conditions (temperature, pH etc.), an oligonucleotide probe will hybridise with a sequence which is not entirely complementary. The degree of base-pairing between the two molecules will be sufficient for them to anneal despite a mis-match. Various approaches are well known in the art for detecting the presence of a mis-match between two annealing nucleic acid molecules.

For instance, RN'ase A cleaves at the site of a mis-match. Cleavage can be detected by electrophoresing test nucleic acid to which the relevant probe or probe has annealed and looking for smaller molecules (i.e. molecules with higher electrophoretic mobility) than the full length probe/test hybrid. Other approaches rely on the use of enzymes such as resolvases or endonucleases.

The presence of differences in sequence of nucleic acid molecules may be detected by means of restriction enzyme digestion, such as in a method of DNA fingerprinting where the restriction pattern produced when one or more restriction enzymes are used to cut a sample of nucleic acid is compared with the pattern obtained when a sample containing the normal gene or a variant or allele is digested with the same enzyme or enzymes.

The presence of absence of a lesion in a promoter or other regulatory sequence may also be assessed by determining the level of mRNA production by transcription or the level of polypeptide production by translation from the mRNA.

A test sample of nucleic acid may be provided for example by extracting nucleic acid from cells, e.g. in

saliva or preferably blood, or for pre-natal testing from the amnion, placenta or foetus itself.

There are various methods for determining the presence or absence in a test sample of a particular polypeptide, such as the polypeptide with the amino acid sequence shown in figure 6 or an amino acid sequence variant or allele thereof.

A sample may be tested for the presence of a binding partner for a specific binding member such as an antibody (or mixture of antibodies), specific for one or more particular variants of the polypeptide shown in figure 6.

A sample may be tested for the presence of a binding partner for a specific binding member such as an antibody (or mixture of antibodies), specific for the polypeptide shown in figure 6.

In such cases, the sample may be tested by being contacted with a specific binding member such as an antibody under appropriate conditions for specific binding, before binding is determined, for instance using a reporter system as discussed. Where a panel of antibodies is used, different reporting labels may be employed for each antibody so that binding of each can be determined.

A specific binding member such as an antibody may be used to isolate and/or purify its binding partner polypeptide from a test sample, to allow for sequence and/or biochemical analysis of the polypeptide to determine whether it has the sequence and/or properties of the polypeptide whose sequence is shown in figure 6, or if it is a mutant or variant form. Amino acid sequence is routine in the art using automated sequencing machines.

Therapeutics.

The DBCCR1 polypeptides, antibodies, peptides and nucleic acid of the invention can be formulated in pharmaceutical compositions. These compositions may comprise, in addition to one of the above substances, a pharmaceutically acceptable excipient, carrier, buffer,

stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration, e.g. oral, intravenous, cutaneous or subcutaneous, nasal, intramuscular, intraperitoneal routes.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

Whether it is a polypeptide, antibody, peptide, nucleic acid molecule, small molecule or other pharmaceutically useful compound according to the present invention that is to be given to an individual, administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of

treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 16th edition, Osol, A. (ed), 1980.

Alternatively, targeting therapies may be used to deliver the active agent more specifically to certain types of cell, by the use of targeting systems such as antibody or cell specific ligands. Targeting may be desirable for a variety of reasons; for example if the agent is unacceptably toxic, or if it would otherwise require too high a dosage, or if it would not otherwise be able to enter the target cells.

Instead of administering these agents directly, they could be produced in the target cells by expression from an encoding gene introduced into the cells, eg in a viral vector (a variant of the VDEPT technique - see below). The vector could be targeted to the specific cells to be treated, or it could contain regulatory elements which are switched on more or less selectively by the target cells.

Alternatively, the agent could be administered in a precursor form, for conversion to the active form by an activating agent produced in, or targeted to, the cells to be treated. This type of approach is sometimes known as ADEPT or VDEPT; the former involving targeting the activating agent to the cells by conjugation to a cell-specific antibody, while the latter involves producing the activating agent, eg an enzyme, in a vector by expression from encoding DNA in a viral vector (see for example, EP-A-415731 and WO 90/07936).

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

Methods of Gene Therapy.

As a further alternative, the nucleic acid encoded the authentic biologically active DBCCR1 polypeptide could be used in a method of gene therapy, to treat a patient who is unable to synthesize the active polypeptide or unable to synthesize it at the normal level, thereby providing the tumour suppressing or growth inhibiting effect provided by wild-type DBCCR1, and thereby suppressing the occurrence of cancer and/or reduce the size or extent of existing cancer in the target cells.

Vectors such as viral vectors have been used in the prior art to introduce genes into a wide variety of different target cells. Typically the vectors are exposed to the target cells so that transfection can take place in a sufficient proportion of the cells to provide a useful therapeutic or prophylactic effect from the expression of the desired polypeptide. The transfected nucleic acid may be permanently incorporated into the genome of each of the targeted tumour cells, providing long lasting effect, or alternatively the treatment may have to be repeated periodically.

A variety of vectors, both viral vectors and plasmid vectors, are known in the art, see US Patent No. 5,252,479 and WO 93/07282. In particular, a number of viruses have been used as gene transfer vectors, including papovaviruses, such as SV40, vaccinia virus, herpesviruses, including HSV and EBV, and retroviruses. Many gene therapy protocols in the prior art have used disabled murine retroviruses.

As an alternative to the use of viral vectors other known methods of introducing nucleic acid into cells includes electroporation, calcium phosphate co-precipitation, mechanical techniques such as microinjection, transfer mediated by liposomes and direct DNA uptake and receptor-mediated DNA transfer.

As mentioned above, the aim of gene therapy using nucleic acid encoding the DBCCR1 polypeptide, or an active

portion thereof, is to increase the amount of the expression product of the nucleic acid in cells in which the level of the wild-type DBCCR1 polypeptide is absent or present only at reduced levels. Such treatment may be therapeutic in the treatment of cells which are already cancerous.

Gene transfer techniques which selectively target the DBCCR1 nucleic acid to bladder cells are preferred. Examples of this included receptor-mediated gene transfer, in which the nucleic acid is linked to a protein ligand via polylysine, with the ligand being specific for a receptor present on the surface of the target cells.

Methods of Screening for Drugs.

A polypeptide according to the present invention may be used in screening for molecules which affect or modulate its activity or function. Such molecules may be useful in a therapeutic (possibly including prophylactic) context.

It is well known that pharmaceutical research leading to the identification of a new drug may involve the screening of very large numbers of candidate substances, both before and even after a lead compound has been found. This is one factor which makes pharmaceutical research very expensive and time-consuming. Means for assisting in the screening process can have considerable commercial importance and utility. Such means for screening for substances potentially useful in treating or preventing cancer is provided by polypeptides according to the present invention. Substances identified as modulators of the polypeptide represent an advance in the fight against cancer since they provide basis for design and investigation of therapeutics for in vivo use.

A method of screening for a substance which modulates activity of a polypeptide may include contacting one or more test substances with the polypeptide in a suitable reaction medium, testing the activity of the treated polypeptide and comparing that activity with the activity

of the polypeptide in comparable reaction medium untreated with the test substance or substances. A difference in activity between the treated and untreated polypeptides is indicative of a modulating effect of the relevant test substance or substances.

Combinatorial library technology provides an efficient way of testing a potentially vast number of different substances for ability to modulate activity of a polypeptide. Such libraries and their use are known in the art. The use of peptide libraries is preferred.

Prior to or as well as being screened for modulation of activity, test substances may be screened for ability to interact with the polypeptide, e.g. in a yeast two-hybrid system (which requires that both the polypeptide and the test substance can be expressed in yeast from encoding nucleic acid). This may be used as a coarse screen prior to testing a substance for actual ability to modulate activity of the polypeptide. Alternatively, the screen could be used to screen test substances for binding to a DBCCR1 specific binding partner, to find mimetics of the DBCCR1 polypeptide, e.g. for testing as cancer therapeutics.

Following identification of a substance which modulates or affects polypeptide activity, the substance may be investigated further. Furthermore, it may be manufactured and/or used in preparation, i.e. manufacture or formulation, of a composition such as a medicament, pharmaceutical composition or drug. These may be administered to individuals.

Thus, the present invention extends in various aspects not only to a substance identified using a nucleic acid molecule as a modulator of polypeptide activity, in accordance with what is disclosed herein, but also a pharmaceutical composition, medicament, drug or other composition comprising such a substance, a method comprising administration of such a composition to a patient, e.g. for treatment (which may include preventative

treatment) of cancer, use of such a substance in manufacture of a composition for administration, e.g. for treatment of cancer, and a method of making a pharmaceutical composition comprising admixing such a substance with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally other ingredients.

A substance identified using as a modulator of polypeptide function may be peptide or non-peptide in nature. Non-peptide "small molecules" are often preferred for many *in vivo* pharmaceutical uses. Accordingly, a mimetic or mimic of the substance (particularly if a peptide) may be designed for pharmaceutical use.

The designing of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. This might be desirable where the active compound is difficult or expensive to synthesise or where it is unsuitable for a particular method of administration, e.g. peptides are unsuitable active agents for oral compositions as they tend to be quickly degraded by proteases in the alimentary canal. Mimetic design, synthesis and testing is generally used to avoid randomly screening large number of molecules for a target property.

There are several steps commonly taken in the design of a mimetic from a compound having a given target property. Firstly, the particular parts of the compound that are critical and/or important in determining the target property are determined. In the case of a peptide, this can be done by systematically varying the amino acid residues in the peptide, e.g. by substituting each residue in turn. Alanine scans of peptide are commonly used to refine such peptide motifs. These parts or residues constituting the active region of the compound are known as its "pharmacophore".

Once the pharmacophore has been found, its structure is modelled to according its physical properties, eg stereochemistry, bonding, size and/or charge, using data

from a range of sources, eg spectroscopic techniques, X-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling process.

In a variant of this approach, the three-dimensional structure of the ligand and its binding partner are modelled. This can be especially useful where the ligand and/or binding partner change conformation on binding, allowing the model to take account of this in the design of the mimetic.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to it can conveniently be selected so that the mimetic is easy to synthesise, is likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the biological activity of the lead compound. Alternatively, where the mimetic is peptide based, further stability can be achieved by cyclising the peptide, increasing its rigidity. The mimetic or mimetics found by this approach can then be screened to see whether they have the target property, or to what extent they exhibit it. Further optimisation or modification can then be carried out to arrive at one or more final mimetics for *in vivo* or clinical testing.

Screening for Substances Affecting DBCCR1 Expression.

The present invention also provides the use of all or part of the nucleic acid sequence of the DBCRR1 promoter region set out in figure 11 in methods of screening for substances which modulate the activity of the promoter and increase or decrease the level of DBCCR1 expression.

"Promoter activity" is used to refer to ability to initiate transcription. The level of promoter activity is quantifiable for instance by assessment of the amount of mRNA produced by transcription from the promoter or by

assessment of the amount of protein product produced by translation of mRNA produced by transcription from the promoter. The amount of a specific mRNA present in an expression system may be determined for example using specific oligonucleotides which are able to hybridise with the mRNA and which are labelled or may be used in a specific amplification reaction such as the polymerase chain reaction. Use of a reporter gene facilitates determination of promoter activity by reference to protein production.

Further provided by the present invention is a nucleic acid construct comprising a DBCCR1 promoter region set out in figure 11 or a fragment thereof able to promote transcription, operably linked to a heterologous gene, e.g. a coding sequence. A "heterologous" or "exogenous" gene is generally not a modified form of DBCCR1 Generally, the gene may be transcribed into mRNA which may be translated into a peptide or polypeptide product which may be detected and preferably quantitated following expression. A gene whose encoded product may be assayed following expression is termed a "reporter gene", i.e. a gene which "reports" on promoter activity.

The reporter gene preferably encodes an enzyme which catalyses a reaction which produces a detectable signal, preferably a visually detectable signal, such as a coloured product. Many examples are known, including β-galactosidase and luciferase. β-galactosidase activity may be assayed by production of blue colour on substrate, the assay being by eye or by use of a spectrophotometer to measure absorbance. Fluorescence, for example that produced as a result of luciferase activity, may be quantitated using a spectrophotometer. Radioactive assays may be used, for instance using chloramphenicol acetyltransferase, which may also be used in non-radioactive assays. The presence and/or amount of gene product resulting from expression from the reporter gene may be determined using a molecule able to bind the

product, such as an antibody or fragment thereof. The binding molecule may be labelled directly or indirectly using any standard technique.

Those skilled in the art are well aware of a multitude of possible reporter genes and assay techniques which may be used to determine gene activity. Any suitable reporter/assay may be used and it should be appreciated that no particular choice is essential to or a limitation of the present invention.

Nucleic acid constructs comprising a promoter (as disclosed herein) and a heterologous gene (reporter) may be employed in screening for a substance able to modulate activity of the promoter. For therapeutic purposes, e.g. for treatment of cancer, a substance able to up-regulate expression of the promoter directing the expression of DBCCR1 may be sought. A method of screening for ability of a substance to modulate activity of a promoter may comprise contacting an expression system, such as a host cell, containing a nucleic acid construct as herein disclosed with a test or candidate substance and determining expression of the heterologous gene.

The level of expression in the presence of the test substance may be compared with the level of expression in the absence of the test substance. A difference in expression in the presence of the test substance indicates ability of the substance to modulate gene expression. An increase in expression of the heterologous gene compared with expression of another gene not linked to a promoter as disclosed herein indicates specificity of the substance for modulation of the promoter.

A promoter construct may be introduced into a cell line using any technique previously described to produce a stable cell line containing the reporter construct integrated into the genome. The cells may be grown and incubated with test compounds for varying times. The cells may be grown in 96 well plates to facilitate the analysis of large numbers of compounds. The cells may then be

washed and the reporter gene expression analysed. For some reporters, such as luciferase the cells will be lysed then analysed.

Following identification of a substance which modulates or affects promoter activity, the substance may be investigated further. Furthermore, it may be manufactured and/or used in preparation, i.e. manufacture or formulation, of a composition such as a medicament, pharmaceutical composition or drug. These may be administered to individuals.

Materials and Methods

Detection of loss of heterozygosity by microsatellite analysis.

Specimens of 145 TCCs of the bladder and 9 TCCs of the ureter or renal pelvis were obtained with paired blood or normal kidney samples as a source of constitutional normal DNA. DNA from tumour specimens and corresponding normal tissues (peripheral blood or normal kidney) were obtained by proteinase K digestion followed by phenol/chloroform extraction. Adjacent portions of each tumour specimen were subjected to histopathological examination. Tumour stage and grade were classified according to the TNM system and the WHO criteria, respectively. We initially used 31 microsatellite markers mapped to 9q. Nine markers on 9q32-33 are shown in figure 1. The other 22 markers on 9q used were D9S15, D9S153, D9S167, D9S152, D9S201, D9S283, D9S119, D9S12, D9S176, D9S109, D9S127, D9S53, D9S58, D9S105, D9S59, D9S123, D9S282, D9S60, D9S61, ABL, D9S66, and D9S67. To evaluate 9p status in 5 tumours with localised deletion at 9q32-33, we tested D9S199, D9S200, IFNA, D9S1749, D9S126 and D9S171 by multiplex PCR (Williamson et al, 1995). Primer sequences were obtained from the Genome Database. PCR reactions were carried out in 12.5 μl reaction volumes with 5 to 10 ng of genomic DNA as template, 1.0 to 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, 2 pmol of each primer, 1 U of Taq DNA polymerase and buffer

supplied by the manufacturer (Life Technologies). One of each primer pair was end-labelled with ^{32}P. PCR reactions consisted of 26-27 cycles of 1 min at 95°C, 1 min at 55°C, and 1.5 min at 72°C followed by a final elongation. Reaction products were diluted with formamide dye, heat-denatured, and run in 6 % denaturing polyacrylamide gels. Gels were dried and exposed to Fuji XR film and subsequently to a PhosphorImager screen (Molecular Dynamics). Initially, LOH was screened visually for loss of one allele, and cases with 'partial loss' or 'allelic imbalance' were further analysed by the PhosphorImager using the ImageQuant software (Molecular Dynamics). A relative decrease in the intensity of the signal from one tumour allele of more than 40% was scored as LOH. Loci at which new alleles were detected (microsatellite alterations) were considered to be 'not informative'.

Isolation and characterisation of YAC clones.

Two YAC libraries were used for YAC clone isolation and construction of a YAC contig. The ICI YAC library (Anand et al, 1990) was screened by PCR using published primer sequences for D9S258, D9S275, D9S195 and D9S302. From the CEPH YAC library (Alberstson et al, 1990), we obtained and analysed 10 YAC clones that have been shown to be positive for one of 3 microsatellite markers D9S195, D9S258 and D9S275 or shown to be contiguous to positive YACs. High molecular weight DNA from each YAC clone was prepared in agarose blocks as described in Chaplin et al, 1995 and subjected to pulse-field electrophoresis in 1% agarose gels using a contour-clamped homogeneous electric field (CHEF) apparatus (Bio-Rad-CHEF DRTM II system). Typical running conditions were as follows: 60 second pulse time for 15 hours followed by a 90 second pulse time for 9 hours at 200 volts in 0.5 TBE buffer at 14°C. After ethidium bromide staining, gels were blotted onto nylon membranes (Hybond N+, Amersham) using 0.4N NaOH as transfer buffer and each blot was hybridised with total human DNA

^{32}P-labelled by random priming. The size of each YAC was evaluated by using Saccharomyces cerevisae (strain YNN295, BioRad) chromosome and mutimers of lambda-phage (BioRad) as size markers. The size of all CEPH YACs shown in figure 2 was consistent with CEPH data (http://www.ceph.fr/).

Generation of sequence-tagged sites (STSs) from YAC-ends.

STSs from YAC insert ends (YAC-ends) were generated using the modified vectorette-PCR procedure as described by Riley et al and direct DNA sequencing of PCR fragments. Briefly, YAC DNAs were prepared as described Chaplin et al (1995) and three vectorette libraries were prepared for each YAC. YAC DNA was digested with RsaI, AluI, or PvuII, and then ligated with the blunt-end vectorette cassette as described in Riley et al (1990). Using these three vectorette libraries as templates, PCR was performed with 224 primer and a primer 5'-CTACTTGGAGCCACTATCGACTACGCGATC to isolate the left arm of each YAC and with 224 primer and a primer 5'-CTTGCAAGTCTGGGAAGTGAATGGAGACAT to isolate the right arm. The resulting PCR products were electrophoresed in agarose gels and amplified fragments of appropriate size were recovered and used as sequencing templates. Direct sequencing was performed using internal primers 1207 and 368 (Riley et al, 1990) for the left YAC-end fragments and 1208 and 368 for the right YAC-end fragment using a cycle sequencing kit (fmol sequencing system, Promega). Oligonucleotides which can amplify a 81 to 190 bp PCR fragment from each YAC-end sequence were generated (Table 1). The presence or absence of these STSs in each YAC clone was tested at least twice by PCR amplification and agarose gel electrophoresis. DNA from a hybrid cell line GM10611 containing an intact human chromosome 9 in a Chinese hamster background and original YAC clone DNAs were used as positive control templates and normal hamster fibroblast DNA as a negative control template.

Construction of the YAC contig map.

Using microsatellite markers and 10 new YAC-end STSs, a YAC contig map was constructed by PCR based analyses. PCR reactions were performed with 20 ng of YAC DNA or yeast cell pellets washed with 1 x TE in 25 μl reaction volumes using 1 unit of Taq polymerase with 200 μM concentrations of each dNTP and 1.0 to 1.5 mM of $MgCl_2$. PCR reactions were routinely carried using a 'hot-start', in which Taq polymerase was added to the reaction after a 5 minutes denaturing step at 95°C. Thirty to 35 amplification cycles with 95°C for 60s, 50 to 55°C for 60s, and 72°C for 90s were performed.

Uniform resource locators (URLs) for public genome data.

We used the following publicly available data using the World Wide Web, which are incorporated herein by reference.

The Centre d'Etudes du Polymorphisme Humain (CEPH): http://www.ceph.fr/Genethon: http://www.genethon.fr/

The Genome Database: http://gdbwww.gdb.org/gdb

Whitehead Institute for Biomedical Research/MIT Center for Genome Research: http://www.genome.wi.mit.edu

cDNA cloning, 5' RACE and sequencing analysis.

First, we obtained 3 I.M.A.G.E. consortium (Lennon et al., 1996) cDNA clones (28122, 32205, 360922) from the UK Human Genome Mapping Resource Centre and 2 cDNA clones (IB1328 and IB1708) kindly provided by Dr. J. M. Sikela at University of Colorado Health Science Center, which have been shown to contain sequences identical or highly homologous to the EST IB3089. We initially sequenced the clone 360922 which had the longest insert (2241 bp) among these 5 clones and sequenced the 3' end of all these clones. Since the reading frame was open at the 5' end of the clone 360922, we then screened a fetal brain cDNA library to get additional upstream sequences. A set of gridded human fetal brain cDNA library provided by the Resource Center/Primary Database of the German Human Genome

Project (Berlin-Charlottenburg, Germany, formerly called the Reference Library Database) was screened with a 235 bp PCR-generated IB3089 probe which was labelled with ^{32}P-dCTP by an incorporation method. Five positive clones (ICRF: p507K1716, p507I2016, p507L24121, p507F03114, and p507K12270) were identified and obtained from the Resource Center. The longest clone, ICRFp507K12270 was subject to complete DNA sequence analysis. All cDNA nucleotide sequences were determined by a primer walking strategy using the fmol DNA sequencing kit (Promega) on double-stranded plasmid template. Both sense and anti-sense strands were sequenced.

5' RACE was performed to characterize the 5' sequence of the IB3089A gene using a human fetal brain Clontech Marathon cDNA amplification kit according to the manufacture's protocol (Clontech). RACE-PCR was carried out with a primer 5'-CTTCTCTTGCAGATACTGAGGA (nt 1141-1119) and a primer AP1, which was provided in the kit. To test the specificity of the PCR product, the product was electrophoresed on agarose gel, alkali-blotted onto the nylon membrane, and probed with a oligonucleotide probe 5'-GGTAGGTCTCCTGCCAAGCA end-labelled with ^{32}P. A corresponding positive PCR band was excised from agarose gel and cloned into a pGEM-T vector using a pGEM-T kit (Promega). The 5' ends of 8 clones were sequenced using the AP1 primer or pGEM-T vector specific primers. Data base searches were carried out using the GCG software package and the BLAST search service from the UK Human Genome Mapping Project Resource Centre.

Determination and sequencing of exon-intron boundaries.

Exon-intron boundaries and their sequence were determined by the vectorette-PCR method as described (Riley et al., 1990). Briefly, high-molecular-weight DNA of YAC clone 9DC8 was digested with AluI, HpaII, RsaI, PvuII, or EcoRV and ligated with a blunt end vectorette cassette as described (Riley, et al., 1990). Using these 5 vectorette

libraries as templates, PCRs were performed with a vectorette-cassette specific primer '224' (Riley, et al., 1990) and primers used for cDNA sequencing. Amplified PCR fragments with appropriate size were gel-purified from low temperature-melting agarose gels and directly sequenced with an internal vectorette-cassette specific primer '368' (Riley, et al., 1990) and each cDNA sequencing primer used. Obtained sequences were compared with the cDNA sequence and primers were made to amplify each exon-containing genomic fragment. For some exon-intron boundaries, another round of vectorette-PCR was performed using these new primers.

SSCP analysis and sequencing.

Forty primary TCCs of the bladder, which showed loss of heterozygosity on 9q, including 9q32-33 were screened for mutation. These tumours included the 5 tumours with localised LOH at 9q32-33 described previously. All these tumour DNA specimens were proved not to have substantial normal cell contamination because clear loss of one allele was detected in the previous LOH analysis (Habuchi et al., 1995). Tumour DNA and corresponding normal peripheral blood or kidney DNA were extracted with a standard method (Sambrook et al., 1989). Ten pairs of primers were designed to amplify the whole coding region of IB3089A from genomic DNA (Table 3). For exon 8 analysis, 4 pairs of primers were used to cover the coding region. For PCR amplification, 20 ng of tumour DNA was amplified using unlabelled primers (10 μM) in 12.5 μl containing 200 μM of dATP, dGTP and dTTP, 4 μM of dCTP, 1 unit of Taq polymerase (Life Technologies), 0.1 μl of [alpha-32P]dCTP and 1.2-1.5 mM $MgCl_2$. PCR conditions were 95°C for 4 min, 5 cycles of 95°C for 60 s, 60°C for 60s, and 72°C for 90 s, followed by 25 cycles of 95°C for 60 s, 55°C for 60 s, and 72°C for 90. For exon 2, exon 8c and exon 8d, PCR products were treated with restriction enzymes to give appropriate size for the SSCP analysis (Table 3). PCR products were diluted 1:1 with denaturing dye containing formamide, denatured at 90°C

for 3 min, and 2 μl of each sample was loaded on gels. Three gels with different conditions were run at room temperature for 16 hours with 3-10 W constant power. The three gel formulations were the following: 6 % acrylamide (29:1 cross linking of acrylamide to bis-cryamide) with or without 5% glycerol in 0.5X TBE buffer and 0.5x MDE™ (FMC) in 0.6X TBE buffer. The gels were dried and exposed to X-ray film for 1-16 hours.

Tumour and normal DNAs from samples showing variation were re-amplified with corresponding PCR primers. The PCR products were gel purified using low-melting agarose gel and sequenced on both strand with PCR primers using the fmol Sequencing System (Promega).

Southern and Northern analysis.

For Southern analysis, 46 samples of paired normal DNA and tumour (all TCC) DNA were analysed. Of these 46 samples, 22 samples were also tested by SSCP analysis for subtle mutation as described above. 10 μg of paired normal DNA and tumour DNA were digested overnight with TaqI or BamHI (New England Biolab) and transferred to nylon membranes (Hybond-N+, Amersham) using 0.4N NaOH as transfer buffer. About 3.1 kb insert of clone ICRFp507K12270 was excised by EcoRI and NotI double digestion, and the resulting 2 fragments were used as a probe. The filters were hybridized overnight with the probe labelled with 32P by the random primer method at 65°C in buffer containing 5X SSPE (Sambrook et al., 1989). The final washing condition was 0.2X SSC, 0.2% SDS at 65°C. Each blot was re-probed with β-actin probe or D9S7 probe for loading control of each sample. For Northern analysis, human multiple tissue northern blots were purchased (Clontech) and hybridized with the ^{32}P labelled probe as mentioned above using the manufacturer's recommended protocol. For Northern analysis of bladder cancer cell lines, total RNA from 5 bladder cancer cell lines was extracted using 'Total RNA Isolation Reagent' (Advanced Biotechnologies) and poly(A)+ RNA was

then purified using oligo-dT Dynabeads (DYNAL). Two μg of poly(A)+ RNA was fractionated and blotted onto a nylon membrane (Hybond-N, Amersham) with a standard method (Sambrook et al., 1989). The final washing conditions for Northern analysis were 0.2X SSC, 0.2% SDS at 60°C.

Cell lines and reverse transcription (RT)-PCR analysis.

Nine transitional cell carcinoma cell lines (T24, SW1710, 5637, RT4, RT112, 253J, J82, UM-UC-3, 609CR) and 1 bladder squamous cell carcinoma cell line (SCaBER) were propagated in RPMI 1640 media or DMEM media supplemented with 10% fetal bovine serum and antibiotics. Histologically normal urothelial tissues from bladder and ureter were obtained from 2 non-bladder cancer patients and dissected under the microscope. Total RNA from the 10 cell lines and normal urothelium of the bladder or ureter was prepared using 'Total RNA Isolation Reagent' (Advanced Biotechnologies). The GAPDH (glyceraldehyde 3-phosphate dehydrogenase) RT-PCR is used as a control for RNA quality and reverse transriptase reaction. Approximately 2 μg of total RNA was reverse transcribed with a oligo-dT primer using a kit (SuperScript Preamplification System, Life Technologies) according to the manufacturer's protocol. Generated cDNA was diluted with TE (pH 8.0) and cDNA equivalent to 100 ng or 25 ng of total RNA was used as a template for IB3089A or GAPDH in 25 μl reaction volume. The primers for IB3089A were designed in exon 7 and exon 8, giving a 232 bp fragment from cDNA template. Their sequences were 5'-CAACGCACTGCCCGCAAGCTT (sense, nt 1487-1508) and 5'-TGTTCCCGCCTATCACGCAGG (antisense, nt 1718-1698). With these primers, no PCR product was obtained from genomic DNA under the following PCR conditions. Conditions for IB3089A RT-PCR were 95°C for 4 min, 10 cycles of 95°C for 60 sec, 60°C for 60 sec and 72°C for 90 sec, and then 25 cycles of 95°C for 60 sec, 55°C for 60 sec and 72°C for 90 sec, followed by incubation at 72°C for 5 min in 1x Taq polymerase buffer (Life Technologies) with

1.2 mM $MgCl_2$, 200 μM dNTPs and 2 unit Taq polymerase (Life Technologies). Primers for the GAPDH gene were also designed to flank multiple introns giving a 455 bp fragment. Their sequences were 5'-CGAGCCACATCGCTCAGACA (sense) and 5'-TGAGGCTGTTGTCATACTTCTC (antisense). Conditions for GAPDH amplifications were 95°C for 4 min, 30 cycles of 95°C for 60 sec, 55°C for 60 sec and 72°C for 120 sec, followed by incubation at 72°C for 5 min in 1x Taq polymerase buffer with 1.0 mM $MgCl_2$, 200 μM dNTPs and 1 unit Taq polymerase. The PCR products were electrophoresed in 2% agarose gels and transferred to a nylon membrane (Hybond N+, Amersham) using 0.4 N NaOH as transfer buffer. Each blot was hybridized with ^{32}P-end labelled oligonucleotide probes, located in the expected amplified PCR fragments. The sequences of internal oligonucleotide probes were 5'-CTCTAGGCAGCTGGTGGTTG (IB3089A, nt 1559-1539) and 5'-GGCTGAGAACGGGAAGCTTG (GAPDH). The blots were exposed to X-Ray film and then subjected to PhosphorImager analysis (Molecular Dynamics) for semi-quantitation of the RT-PCR products. The ratio between IB3089A and GAPDH were obtained for each sample. All reactions, including the reverse transcription reaction were done at least twice, and all were monitored with the omission of reverse transcriptase except normal bladder and ureter RNA sample, since there was only a limited amount of RNA available and both expected RT-PCR fragments were proved to be not amplified from genomic DNA.

Demethylating drug treatment and methylation analysis.

For 5-aza-2'-deoxycytidine treatment, 2-5x10^5 cells were plated onto 100 mm diameter dishes and were exposed to 5-aza-2'-deoxycytidine (Sigma) at a final concentration of 1 μM for 18 hrs. Media with fresh drug was added after 42 hrs. Total RNA was extracted after a 4 day-exposure to 5-aza-2'-deoxycytidine from drug treated and mock treated cell samples. For Southern analysis of the methylation status of the 5' region of IB3089A, genomic DNA was

isolated from TCC cells (609CR, 5637, 353J, SW1710 and T24) and 10 primary TCC specimens by a standard procedure (Sambrook et al., 1989). Ten μg total genomic DNA was subjected to restriction enzyme digestion with BssHII (100 unit) followed by BamHI (100 unit) or PvuII (100 unit) for 16 hrs under conditions specified by the manufacturer (New England Biolabs). Digests were size fractionated on a 1.2 % agarose gel, transferred onto a nylon membrane (Hybond-N+, Amersham) using 0.4N NaOH. Membranes were hybridized at 65°C in buffer containing 5x SSPE with a IB3089A 5' region probe labelled with 32P-dCTP by the random primer method. The 5' probe was prepared by PCR amplification of a 312 bp cDNA fragment (nt 57-368) using clone ICRFp507K12270 as a template. The probe was labelled by incorporation of ^{32}P-dCTP during a second PCR without 'cold' dCTP using the purified 312 bp PCR fragment as a template. The final washing condition of the filters was 0.2x SCC, 0.2% SDS at 65°C.

Transfection Experiments.

The DBCCR1 gene was inserted into an expression vector under the control of a stong heterologous promoter, with the gene linked to a small epitope tag HA to facilitate antibody detection. This construct was used to transfect mouse 3T3 cells and 2 human bladder tumour cells lines that do not express endogenous DBCCR1 (cell lines 5637 and EJ). The expression of the DBCCR1 gene and immunohistochemistry of the DBCCR1 polypeptide was then determined.

<u>Results</u>

Localization of a candidate region

We analysed 156 TCCs of the bladder and upper urinary tract using 31 microsatellite markers on 9q. These tumours included 26 TCCs which showed retention of heterozygosity at all informative loci examined in a previous study (Keen et al, 1994). LOH at at least one locus on 9q was detected in 87 (56%) of 156 TCCs whereas 69 (44%) showed retention

of heterozygosity at all loci. Seventy-seven (49%) tumours showed LOH at all informative loci on 9q. This frequency of LOH on 9q is an underestimate of the overall frequency in TCC since the present study included a selected group of 26 tumours which showed retention of heterozygosity at all informative 9q loci examined previously (Keen et al 1994). If these cases are not included, 86 (66%) of 130 TCCs showed LOH at at least one locus on 9q. Ten (6%) tumours showed partial deletions on 9q and 5 (3%) of the 10 tumours had LOH at D9S195, which is mapped at 9q32-33, and retention of heterozygosity at all other informative loci on 9q (Fig. 1 and 2). These 5 tumours also showed retention of heterozygosity at all informative 9p21 markers and no homozygous deletion at 9p21 by multiplex PCR analyses (Williamson et al, 1995). The results for the other 5 partial deletions on 9q have been described previously (Habuchi et al, 1995), and further deletion mapping in these 5 tumours did not significantly narrow the localisation of the deleted region (data not shown). In accordance with previous studies, the existence of LOH on 9q was not significantly associated with tumour grade and stage. LOH at at least one locus on 9q was observed in 14 (67%) of 21 grade 1 TCCs, 38 (68%) of 56 grade 2 TCCs, and 23 (61%) of 38 grade 3 TCCs ($p>0.1$, Chi-Square). As for stage, LOH on 9q was found in 26 (72%) of 36 Ta TCCs, 16 (52%) of 31 T1 TCCs, and 27 (69%) of 39 T2 or higher stage TCCs ($p>0.1$, Chi-Square). Interestingly, the 5 tumours with localised LOH at D9S195 were all classified as low grade (grade 1 or 2) superficial (Ta or T1) TCCs (Fig. 1).

For the identification of a candidate tumour suppressor gene at this locus, we then constructed a yeast artificial chromosome (YAC) contig of the deleted region. The Généthon linkage map (http://www.genethon.fr/, March 1996) places both D9S195 and D9S258 1 cM distal to D9S275 at 135 cM from the top of chromosome 9 and another linkage data has placed D9S195 proximal to GSN (27,28). However, the precise order of the markers D9S103, D9S258, D9S275,

D9S195, D9S302 and GSN was not defined clearly. We obtained and analysed 10 YAC clones from the Centre d'Etudes du Polymorphisme Humain (CEPH) (Albertson et al, 1990) known to be positive for D9S195, D9S258, and D9S275. We also screened the ICI YAC library (Anand et al, 1990) with D9S195, D9S258, D9S275, and D9S302 by PCR and we identified 11, 7, and 3 positive clones for D9S195, D9S258, and D9S275 respectively (Fig. 3). Three CEPH YACs were found to contain D9S103 and 3 other CEPH YACs contained GSN (Fig. 3). We could not find a YAC containing D9S302 in the ICI library. We first constructed a YAC contig by PCR analyses using the 5 markers (D9S103, D9S195, D9S258, D9S275, and GSN). To further refine the YAC contig map, we then isolated 10 YAC-end fragments from 5 clones by the vectorette method, and established 10 sequence-tagged sites (STSs) (Table 1). These new STSs were mapped to chromosome 9 by PCR using the human-Chinese hamster ovary hybrid cell line GM10611, which contains an intact human chromosome 9 in a Chinese hamster background and the other YACs. We also identified the marker D9S123 in CEPH YAC 765b11.

Considering the linkage data and CEPH YAC data (http://www.ceph.fr/) indicating that YACs 755g12, 798e3, 767h1 and 765b11 overlap each other, the resulting YAC contig map is shown in Figure 3. Our data indicates the likely order of these markers as (centromere)-D9S275-D9S195-D9S258-D9S103-GSN-(telomere) and YAC 908c11 may contain large internal deletion. However, if we assume that no YACs have an internal deletion, the orientation of the markers from D9S275 to D9S103 (Fig. 3) may be reversed and the likely order is (centromere)-D9S103-D9S258-D9S195-D9S275-GSN-(telomere). In either case, the flanking markers for the deleted region in the five tumours with selective deletions are D9S258 and D9S275 (Fig. 3). Although D9S302 was previously closely linked to D9S195 by linkage analyses, this marker was absent from all the YAC clones analysed.

In order to further define the deleted region, we

tried to locate other published microsatellite markers on this YAC contig map. According to Généthon linkage data and data from the Whitehead Institute-MIT Genome Center (http://www-genome.wi.mit.edu/, Release 11, October 1996), 4 microsatellite markers D9S1848, GGAA-P17524, AFMA239XA9, AFMA239ZE1 have been mapped close to D9S195. Using the YAC contig map, we placed D9S1848 between 852e11-R and 9DC8-R, AFMA239ZE1 between 814c5-L and 12IB1-R, AFMA239XA9 between 12IB1-L and 852e11-L and GGAA-P17524 between 15HD3-R and 814c5-R (Fig. 3). The likely order of these markers is therefore(centromere)-D9S275-D9S1848-AFMA239ZE1-D9S195-AFMA239XA9-GGAA-P17524-D9S258-D9S103-GSN-(telomere) (Fig. 3). Using these markers, we analysed the extent of the deletion in the 5 tumours with localised deletion at 9q32-33 (Fig. 2 and 4). Three tumours (#35, #68, and #121) retained heterozygosity at AFMA239XA9, and all 5 tumours retained heterozygosity at D9S1848. In addition to D9S195, LOH was detected at AFMA239ZE1 in tumours #68 and #102. Since only tumour #121 showed retention of heterozygosity at AFMA239ZE1, the consensus candidate region for a tumour suppressor gene is between D9S1848 and AFMA239XA9 (Fig. 4). This region is covered by a single YAC (852e11) and is considered to be less than 840 kb, if this YAC has neither deletion nor rearrangement (Fig. 3).

Identification and cDNA sequence of a novel gene at 9q32-23.

The results above show that one of candidate tumour suppressor loci on 9q is localized at 9q32-33 and that the critical candidate region is between D9S1848 and AFMA239XA9 (Fig. 5). The candidate region is encompassed by a single CEPH YAC 852e11, whose size is estimated to be 840 kb. As an initial attempt to identify a candidate tumour suppressor gene at this locus, we searched for the presence of expressed sequence tags (ESTs) mapped within or near the region using the YAC contig map. The Whitehead Institute/MIT center for Genome Research data base suggests

that one EST, named IB3089 (GeneBank: T16063), is positive in YAC 852e11. We made primers for EST IB3089 and placed this EST between 9DC8-R and 814c5-L by PCR analysis (Fig. 5). Since this site was within the candidate tumour suppressor region, IB3089 represents a good candidate gene. The DNA sequence of IB3089 (317 nucleotides) was used to search the GeneBank and dbEST databases using the BLAST search program (Altschul et al., 1990). The search demonstrated that several ESTs (GeneBank: R42707 (345 nts), T15661 (375 nts), T15475 (359 nts), AA011030 (442 nts), Z41452 (312 nts), F08986 (309 nts), R40799 (214nts)) contain nearly identical sequences to this EST. We obtained 3 original cDNA I.M.A.G.E. Consortium (Lennon et al., 1996) clones (28122, 32205, 360922) from the UK Human Genome Mapping Project Resource Centre and another 2 original cDNA clones (IB1328 for T15475, IB1708 for T15661) from Dr. J. M. Sikela. We then sequenced the insert of the longest clone 360922 among these 5 clones and the 3' end of all these clones. Since the preliminary sequence analysis of the clone 360922 indicated that the 5' end of this clone was not complete, we then screened the gridded fetal brain cDNA library provided by the Resource Center/Primary Database of the German Human Genome Project (Berlin-Charlottenburg, Germany) with the PCR-generated probe IB3089. Five positive clones were identified and the longest clone (ICRFp507K12270) had an insert of about 3.1 kb. The complete sequence of this clone was determined (Fig. 6). To characterize the 5' end of this gene further, 5' rapid amplification of cDNA ends (RACE) analysis using fetal brain cDNA (Marathon-Ready cDNA, Clontech) was performed. Sequence analysis of multiple 5'-RACE clones gave only 10 base pairs of additional sequence information to the 5' of clone ICRFp507K12270 (Fig. 6). At the 3' end, the longest sequence was obtained from clones 28122 and IB1708 and these clones had 4 bp of additional 3' cDNA sequence to clone ICRFp507K12270 (Fig. 6). The overall cDNA sequence represented a new gene subsequently named

IB3089A (IB3089 derived gene, A). The cDNA sequence of the IB3089A gene consists of 3,158 base pairs and defines a single long 2,283 nucleotide open reading frame of 761-amino acids with a calculated molecular weight of 88,689. The putative translation initiation site is at nucleotide 419 and the sequence surrounding the site conforms to Kozak rules (Kozak, 1987; Kozak, 1991). The sequence 5' to the proposed initiation methionine has stop codons in all three open reading frames. Comparison of the complete IB3089A cDNA nucleotide sequence with GeneBank sequences and dbEST using the BLAST algorithm showed significant homology to several ESTs which were shown to be almost identical to EST IB3089 or to ESTs from the 5' end sequence of the corresponding clones. In addition, the search showed that 2 other 3' end ESTs (H10959 and R42933) are almost identical to the region spanning nt 2330-2790. These ESTs might be derived from mRNA using an alternative polyadenylation site or caused by an artifact in constructing cDNA library. Furthermore, the BLAST search showed that the nt 1-400 and 2580-2720 are homologous to mouse brain ESTs (GB: W50233, W64061) (p=3.3e-114 and 1.6e-39). The result indicates that the IB3089A gene is conserved between human and rodents. However, a search of the predicted IB3089A protein with the SwissProt, the ProDom (release ProDom33) (Sonnhammer and Kahn, 1994) and the SBASE (Pongor et al., 1994) databases using the BLAST algorithm revealed no significant homology with known amino acid sequences or domains. Searching the PROSITE database (release 12.2) (Bairoch, 1992) with the IB3089A protein sequence identified 7 putative N-glycosylation sites, 4 putative N-myristoylation sites and 30 putative phosphorylation sites by several protein kinase. This indicated that the protein may encode a secreted or plasma membrane protein.

Genomic structure of IB3089A and mutation analysis in primary TCCs.

The nearly full-length insert of the clone ICRFp507K12270 was hybridized with blotted YAC (852e11, 9DC8 and 15HD1) DNAs digested with BamHI. This sequence hybridized with 852e11, 9DC8 and 15HD1. Since the EST IB3089, which represents the 3' end of the IB3089A is located between 9DC8-R and 814c5-L, the 5' end of the gene is considered to be located telomeric to the 3' end (Fig. 5). This result was confirmed by hybridization with a specific 5' probe (nt 56-368) which was later used for methylation analysis. Since this probe hybridized with YACs 852e11, 9DC8 and 15HD3 with identical band size, the 5' end of IB3089A was considered to be located between STSs 15HD3-L and 9DC8-L Fig. 5). PCR amplification and sequence analyses using the vectorette method from the 5 9DC8 YAC vectorette libraries identified 8 exons from 106 bp to 1595 bp in length (Table 3). The initiation codon is located in exon 2. We then made 10 sets of primer pairs to amplify all the coding sequence of IB3089A from genomic DNA (Table 3). Using these primers, we screened 40 primary TCCs of the bladder which showed LOH at 9q32-33 for mutations using single-strand conformation polymorphism (SSCP) analysis. These tumours included the 5 tumours with localised deletion at 9q32-33 described previously above. Although several variant bands were detected, all these variant bands were considered to be normal sequence polymorphisms by subsequent sequence analysis of both normal (peripheral blood) DNA and tumour DNA. The polymorphisms identified were T1036C (Ser to Ser) in 9/40 (22.5%) cases, C2044A (Ile to Ile) in 1/40 (2.5%) cases and T2642C (Leu to Leu) in 1/40 (2.5%) cases. To examine whether any genomic structural alterations or homozygous deletions exist in primary bladder cancers, we performed Southern blot analysis in 46 paired normal and tumour DNAs digested with either TaqI or BamHI. Twenty-two of the 46 samples were also analysed for mutation by the SSCP analysis as described. Southern blot analysis using the insert fragment of clone ICRFp507K12270 as a probe demonstrated

neither variant bands nor homozygous deletion in these 46 tumours (data not shown).

Homozygous deletion of the DBCCR1 gene in a bladder tumour

More recently, we have identified a bladder tumour which shows deletion of both alleles in the region of DBCCR1. To detect homozygous deletion, we carried out duplex PCR using primers for loci in the critical region of deletion and primers for a locus on 8p which showed no LOH in the tumour concerned. Using restricted numbers of PCR cycles, homozygous deletion was identified as a faint signal showing apparent retention of heterozygosity compared with a strong signal from the control primers and from markers in the region which retained one allele. Using this approach (Williamson et al, 1995), we mapped the region of homozygous deletion to the interval between D9S275 and D9S258. Homozygous deletions are strongly indicative that a tumour suppressor gene is nearby and since our work has identified no gene other than DBCCR1 within this region of homozygous deletion, this provides additional evidence that DBCCR1 is the critical gene.

Expression of IB3089A in Normal Tissues and Bladder Cancer Cells.

We examined the expression of the IB3089A gene in normal tissues and bladder cancer cells. Northern blot analysis using mRNAs from a variety of different normal human tissues detected a single major band corresponding to 3.0-3.5 kb in size in multiple tissues (heart, brain, lung, skeletal muscle, kidney, thymus, prostate, testis, small intestine) (Fig. 7). Significantly high expression was detected in brain tissue and a minor 8.5 kb mRNA was observed along with the dominant 3.0-3.5 kb band (Fig. 7). Since the size of the major band corresponds to the 3.2 kb IB3089A cDNA sequence presented herein (Fig. 6), the cDNA sequence is considered to be full length. The minor 8.5 kb signal in brain might come from a different gene with

similar sequence or alternatively spliced mRNA or the use of an alternative promoter or an alternative polyadenylation site.

We then assessed the mRNA expression in 5 bladder cancer cell lines (253J, 5637, RT4, UMUC-3, 609CR) by Northern analysis. By loading 2 ug of poly(A)+ mRNA in each sample, we could not detect any obvious band in these cell lines (data not shown), indicating that IB3089A is not expressed or expressed in very low levels in bladder cancer cells. To enhance the sensitivity of the mRNA expression analysis, we then carried out RT-PCR analysis of IB3089A in 10 bladder cancer cell lines and normal urothelium from bladder and ureter. Since preliminary RT-PCR analysis showed that the expression level was insufficient to be evaluated by ethidium bromide staining of the electrophoresed PCR products, we performed Southern blot hybridization of the PCR products. Southern hybridization of a ^{32}P-labelled internal specific oligonucletide probe with the RT-PCR products revealed that IB3089A is expressed in normal urothelium, bladder cancer cell lines 253J, SW1710, J82, UM-UC-3 and RT112 (Fig. 8). Although not strictly quantitative, expression in SW1710 and 253J was higher than in normal urothelium and in RT112 was lower. Expression was not detected in 5 bladder cancer cell lines (5637, T24, RT4, SCaBER and 609CR) even when the blot was exposed for much longer or when 40 cycles of PCR were used (Fig. 8). These results indicated that expression of IB3089A is frequently down regulated or silenced in bladder cancer cells.

Methylation of IB3089A and Induction of IB3089A Expression by a Demethylating Agent.

It has been proposed that aberrant hypermethylation of CpG islands near the 5′ ends of some tumour suppressor genes is one of the mechanisms for their inactivation (Jones, 1996 for review). Although extensive genomic sequence data of the 5′ region of IB3089A has not been

obtained, at least the 368 bp first exon conforms to the criteria for a CpG island defined by Gardiner-Garden and Frommer (Gardiner-Garden and Frommer, 1987), since exon 1 has a 66.6% G+C content (>50%) and the observed/expected CpG dinucleotide ratio is 0.91 (>0.6). Therefore, we investigated the possibility that the reduced expression of IB3089A was caused by hypermethylation of this CpG island. Bladder cancer cell lines and 10 primary TCCs of the bladder were examined. Exon 1 contains a single BssHII site (nt 37-42, Fig. 6), which is also a characteristic of the CpG island (Lindsay and Bird, 1987). We tested the methylation status of the BssHII site since this enzyme is CpG methylation sensitive. We prepared a 312 bp exon 1 specific probe (nt 57-368) that hybridizes the 3' side of the BssHII site (Fig. 9). Restriction mapping analyses using YACs indicated that there is another BssHII site in intron 1 at 0.6 kb from the BssHII site in exon 1 and these 2 BssHII sites are both located within a 1.7 kb BamHI fragment (Fig. 9A). Co-digestion with BamHI and BssHII in normal tissues gave a major 0.6 kb band with complete disappearance of the 1.7 kb band (Fig. 9). However, the 0.6 kb band was not detected in 3 of 5 bladder cancer cell lines and an additional 2 bands (1.0 kb and 1.3 kb) were visualized with varying degrees of retention of the 1.7 kb band in all cell lines. (Fig. 9B). The methylation status of the 2 BssHII sites did not correlate absolutely with the expression level of IB3089A, since the sites are almost unmethylated non-expressing cell line 5637 and both sites were methylated in the expressing cell line 253J. Three of 10 (30%) primary TCCs showed obvious hypermethylation of these sites with the retention of the 1.7 kb band and the presence of 1.0 kb and 1.3 kb bands, and another tumour showed weak bands with 1.0 kb and 1.3 kb in size (Fig. 9B). The results indicated that these 2 BssHII sites were frequently hypermethylated in bladder cell lines and primary TCCs. Furthermore, the 1.0 kb aand 1.3 kb bands were faintly visualized in all normal cells (Fig. 9B),

indicating that these BssHII sites may in methylated in a small fraction of normal cells. It is unlikely that these bands came from partial digestion by BssHII, since these bands were not visualized in some primary TCC samples with over loaded amount of DNA (see Fig. 9B, lane 6). Therefore, it might be a aging-related hypermethylation observed, for example, in the estrogen receptor gene in colonic mucosa (Issa et al., 1994). These results were also confirmed by PvuII digestion in normal tissues and bladder cancer cell lines with or without additional BssHII digestion.

We then tested whether IB3089A de novo expression could be obtained by culturing the non-expressing cell lines, T24, 609CR and 5637, in the presence of methylation inhibitor 5-aza-2'-deoxycytidine. Induction of IB3089A expression was observed in all three cell lines after a 4 day exposure to the drug (Fig. 10). Thus, demethylation was considered to be sufficient to relax the silencing of the IB3089A expression. This result further supports the involvement of hypermethylation-associated silencing of the gene.

Promoter Sequence of DBCCR1

A cosmid clone 50A8 containing DBCCR1 was identified by screening the chromosome 9 cosmid library LL09NC01P with a probe derived from the left arm of YAC 9DC8. The promoter sequence of DBCCR1 was determined by sequencing the cosmid directly upstream of the transcription start site, initially using an antisense primer derived from the cDNA sequence and subsequently using primers derived from the new sequence. This promoter sequence of 1632bp is shown in figure 11.

Transfection Experiments.

Transfection of Swiss CT3 cells and human bladder tumour cell lines 5637 and EJ with a mammalian expression contruct containing the DBCCR1 gene led to a marked

reduction in the number and size of stable transfectant colonies as compared to cells transfected with vector alone. The colonies that were obtained after transfection with DBCCR1 showed a very limited proliferative capacity, indicating that DBCCR1 has a negative regulatory function when overexpressed, e.g. it causes growth inhibition. Immunohistochemistry with the detecting antibody indicated a cytoplasmic location of the gene product.

Discussion

Frequent occurrence of monosomy 9 or LOH at all informative loci in TCC indicates that inactivation of multiple tumour suppressors on chromosome 9 may occur during the genesis and progression of TCCs. In support of this theory, recent studies by the present inventors and Simoneau et al have shown that at least two tumour suppressor loci, one at 9q13-31 and another at 9q34, may exist on chromosome 9 in addition to that at 9p21. The results above show 5 tumours with localised deletion at 9q32-33, indicating the presence of another candidate tumour suppressor locus for TCC in this region. Although the frequency of localised deletion at this locus is not high (3%), there are several reasons to predict the presence of an important tumour suppressor gene in this region.

First, accumulating data from several deletion mapping studies in TCC have shown that partial deletions on 9q mostly encompass this region, although localised deletion at 9q34 or at 9q13-31 is also found at low frequency. So far, we have found 13 TCCs with partial deletion on 9q and the deletion in only a single tumour does not include the region defined in this study.

Second, since LOH involving the entire long arm of chromosome 9 is found in more than 50% of TCCs, inactivation of a tumour suppressor at 9q32-33 and/or other tumour suppressor(s) on 9q may occur in a substantial portion of TCCs with LOH on the whole of 9q. Furthermore,

a recent study of microcell-mediated transfer of normal chromosome 9 into bladder cancer cells also indicates the presence of more than one tumour suppressor locus on 9q and one candidate region encompasses the region defined in this study. Interestingly, all 5 TCCs with localised deletion at 9q32-33 were low grade superficial tumours (Fig. 1). Although we have not sequenced p16 and p15 genes in these tumours, all these tumours showed retention of heterozygosity at 9p21 and no homozygous deletion by multiplex PCR analyses. Therefore, it may be that inactivation of a tumour suppressor located on 9q32-33 sometimes occurs in these low grade superficial tumours without inactivation of other tumour suppressors on chromosome 9. However, this is likely to be a rare event since LOH at all loci on chromosome 9 or monosomy 9 is frequently found even in low grade low stage TCCs. The identification of all relevant tumour suppressor(s) on chromosome 9, including the gene identified in this application, will be required to define the roles of partial or total hemizygous loss of chromosome 9 in the genesis and progression of TCC.

LOH involving 9q has been reported in other types of human cancer, including squamous cell carcinoma of the head and neck (Ah-See et al, 1994), squamous cell carcinoma of the skin (Quinn et al, 1994), ovarian cancer (Schultz et al, 1995, Devlin et al, 1996), renal cell carcinoma (Cairns et al, 1995) and oesophageal cancer (Miura et al, 1995). The localised deleted regions reported in these other cancers encompass the region at 9q32-33 described here. One of the candidate tumour suppressor loci in ovarian cancer has been mapped between HXB at 9q32 and ASS1 at 9q34.1 (Schultz et al, Devlin et al), which encompasses this region. Partial deletions telomeric to HXB and telomeric to D9S127 at 9q22.1-32 were reported in renal cell carcinoma and head and neck squamous cell carcinoma, respectively (Ah-See et al, Cairns et al). Although a candidate tumour suppressor locus in oesophageal cancer has

been mapped at 9q31-32, which is reported to be distinct from the region reported here, many partial deletions in oesophageal cancer also involve the region at 9q32-33.

The present inventors have also identified a novel gene, IB3089A, located within the candidate tumour suppressor region at 9q32-33 defined above. Since LOH encompassing at 9q32-33 is detected in approximately 60% of TCC, somatic alteration or mutation leading to the inactivation of the gene product might be expected in a considerable proportion of TCCs if the gene is a target for LOH at this locus. To date, several tumour suppressor genes have been identified by means of positional cloning and have been shown to be mutated or homozygously deleted in certain percentage of various tumours. However, we could not detect any somatic mutation by SSCP analysis in 40 primary TCCs with LOH at 9q32-33. Since we could identify several polymorphisms in our mutation screening, it is unlikely that we missed a considerable number of somatic mutation in the 40 TCCs. Considering the further result showing no aberrant findings in Southern analysis of 46 TCCs, we conclude that the IB3089A is not or a rare target of somatic mutation or gross genetic alteration to both alleles in TCC.

Alterations of the pattern of DNA methylation have been recognized as consistent molecular changes in wide variety of human cancer (Jones, 1996 for review). Recently, accumulating evidence suggest that aberrant hypermethylation of CpG island in or around the 5' regulatory areas of genes is one of the inactivation mechanisms of tumour suppressor gene. Aberrant methylation and silencing of controversial tumour suppressor genes, such as p15 (Herman et al., 1996), estrogen receptor (Issa et al., 1994) and E-cadherin (Yoshiura et al., 1995), as well as well-defined tumour suppressor genes, Rb (Greger et al., 1989; Sakai et al., 1991), VHL (Herman et al., 1994) and p16 (Merlo et al., 1995; Herman et al., 1995; Gonzalez-Zulueta et al., 1995), have been reported.

It is believed that CpG islands generally remain free of methylation in somatic cells except for inactive X-chromosome genes or imprinted genes (Cross and Bird, 1995; Jones, 1996). In agreement with this rule, the BssHII sites in exon 1 and intron 1 of IB 3089A were nearly totally unmethylated in normal human placenta and peripheral blood, indicating that the CpG island of IB3089A is not methylated in normal somatic cells including normal urothelial transitional cells. Partial or complete hypermethylation of the BssHII sites in exon 1 and intron 1 was observed in all 5 bladder cell lines tested and 4 of 10 (40%) of primary TCCs with varying degrees irrespective of the expression status of IB3089A. These results suggest that the 5' region of IB3089A is a frequent target for aberrant hypermethylation in bladder cancer.

It is interesting to note that some known hot spots for LOH in cancers are also the frequent targets for aberrant hypermethylation (de Bustros et al., 1988; Nagatake et al., 1996; MacGrogan et al., 1996). The association may represent the gene silencing of the target tumour suppressor gene in each candidate tumour suppressor region. Otherwise, the results raise the possibility that hypermethylation of CpG islands may induce localized genome instability of the region that would induce LOH involving the region. Although the methylation status of the BssHII sites in exon 1 and intron 1 did not correlate absolutely with the expression of IB3089A mRNA, it does suggest that repression of IB3089A in these cell lines was caused by hypermethylation of some CpG site(s) of the IB3089A regulatory region since the mRNA re-expression was induced by 5-aza-2'-deoxycytidine treatment in non-expressing bladder cancer cell lines. The presence of a BssHII site in intron 1 indicates that the CpG island of the exon 1 of IB3089A extends 3' to exon 1. The discrepancy between the IB3089A expression status and the 2 BssHII methylation status in bladder cancer cell lines indicates that hypermethylation of CpG site(s) other than these 2 BssHII

sites might be crucial for repression of expression. The promoter sequence now determined contains many CpG dinucleotides which may be methylated and show a more clear cut association with expression.

As IB3089A is predominantly expressed in the adult brain and all the ESTs or cDNA clones corresponding the IB3089A originated from fetal or infant brain or adult retina cDNA libraries, one of the its main function may be important in neuronal activities. Like the colorectal tumour suppressor DCC (Hedrick et al., 1994), which encodes a netrin receptor required for axon guidance (Keino-Masu et al., 1997), the IB3089A protein might have an important function in both neuronal cells and non-neuronal cells.

In summary, we have identified a novel gene, IB3089A, in a bladder cancer-tumour suppressor region at 9q32-33. Expression of IB3089A was frequently down regulated in bladder cancer cells in vitro and thus can be reversed by treatment with a demethylating agent. Our results indicate that downregulation of IB3089A expression in bladder caner cells lines and bladder tumours is due to hypermethylation of the 5' region of the gene. These findings may be applicable to other types of human cancer associated with deletions and/or hypermethylation in this region.

References:

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TABLE 1

PCR primers and conditions for new YAC-end sequence-tagged sites (STSs)

YAC-end	primer sequence (5'-)	size (bp)	annealing temp.(ºC)	$MgCl_2$
814c5-L	1:GTACCTTAATAGCCTACAAGAC 2:CGGAATTCAATTCAGCTAAGTC	133	55	1.0
814c5-R	1:TTCATGACCCTGTACTGTTTGC 2:CACTTTGGTGATAACCTCCATTC	137	55	1.0
852e11-L	1:GAACTTTACATGCCGATAGACTTTG 2:GAATTCTTGGCTCTTGTACTCTGT	113	55	1.0
852e11-R	1:TGGGCTGCACAATTAGAACGTG 2:AGGGAGAATTTGACAGCGAGAT	144	55	1.0
9DC8-L	1:GCAATCAGCCTGAATGCAGGCT 2:ACTAGGGATCTGCATTGCTGAT	162	50	1.0
9DC8-R	1:GAATTCAGGAGCCATGTGGAAT 2:AGGGAAACTAGTCTCAGAGAACT	140	55	1.5
12IB1-L	1:CCATGGTTTGAACTGCAGTGTA 2:AGGGGTATGGTCATGAAGGATA	190	55	1.0
12IB1-R	1:TTGACTGGAGAGCTAGTTTGCC 2:ACTGGATTCCTAGAGTGTATGTC	168	55	1.0
15HD3-L	1:ATCTAGCCTTGCAAAGGTCCTAC 2:AGCTTGTAATCTGTAACGTGGCT	113	57	1.0
15HD3-R	1:CCTTAATGCATACAGATCAATGCC 2:AAGGACAAGTGGATCCTACCAT	81	55	1.0

TABLE 2

Intron/exon Splice Sequences of the *IB3089A* Gene

Intron No.	Acceptor sequence	exon No. (size,bp)	Doner sequence	Intron No.
		1 (≥368)	TTGACTCCAG/gtaaggcggcgccgg	1
1	ttcttttcttctatag/GACTCCACAG	2 (268)	AAATATACAG/gtaagacctgggcta	2
2	atttctcctgcccag/GGAGTTTGCC	3 (191)	ACATTGGGAG/gtaggtcagcagcca	3
3	ttctttcccatccag/GGGAGGAGGC	4 (170)	AGCAATCAAG/gtacagctccggggt	4
4	tgatatctactgcag/GTCACAGAGA	5 (106)	CACCTTCAAG/gtaggaagccagtgg	5
5	gctattctcacacag/GTCTTCAGAT	6 (237)	GAGAATTCAG/gtagagagtctcact	6
6	tttgtctctcactag/ATGAGTTTAA	7 (223)	CTAGAGAGAG/gtaagtgttgccacc	7
7	tcctttatctcccag/GACAATTCAG	8 (1595)		

Intron sequences and exon sequences are shown in lower case letters and upper case letters, respectively.

TABLE 3

PCR Primers Used in SSCP Analysis To Amplify *IB3089A* Coding Regions From Genomic DNA

Pimer	Seqeunce (5'—›3') (foward, reverse)	Product length (bp)
SSCPex2	CTCCACAGAATGGAGCAAGA CAAGGGCTATTTAGCCCAGGT	294 (MspI, 38*+258)
SSCPex3	GGTAAGTTGAGAAGTGTTGCTCA TCTGGCCCAGTGGCTGCTGA	253
SSCPex4	GAAAGCTCCACAAACCTTCTTAT GAGAACCTGTCGCCCAAATCCA	249
SSCPex5	TAGACAAGGACTGCTGTCTCAC CCATGATGTTTCCCCCAACTGA	203
SSCPex6	CTGTTTTCCCTTCTTGCTATTCTCA ACTCATGCAGTGGCACTGAGT	300
SSCPex7	GTGTTATCATGCTTAGCCCTTCT TAGGTGCCTGCAGAGGTGGA	308
SSCPex8A	CATGGTAGCTAACCTTCTCCAAT TAGCCCTTGTTGCAGGAGCC	256
SSCPex8B	GTGCAGCCTGGCCAACATCT CGGATCTCGTTGCTGATGAAG	219
SSCPex8C	ATGGACTCACGCCTCTACGT GCCCTGGCCAGTCTCATTTC	426 (HincII, 210+216)
SSCPex8D	CTCGGCTACCTACCCTACTG TGGCAAGGAGTCCCGGGTTA	436 (MspI, 269+156+15*)

Note. For exon 2, exon 8C, and exon 8D-SSCP analyses, PCR fragments were digested with MspI (exon 2 and exon 8D) or HincII (exon 8C) before gel-electrophoresis. The smallest fragment in exon 2 and exon 8D (indicated by *) is located in non-coding regions.

Claims:

1. An isolated nucleic acid molecule comprising the nucleotide sequence as set out in figure 6, or alleles thereof.

2. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide having the amino acid sequence as set out in figure 6.

3. The nucleic acid molecule of claim 1 or claim 2 which includes one or more of the polymorphisms selected from the group consisting of T1036C, C2044A and T2642C.

4. An isolated nucleic acid molecule encoding a polypeptide having a 80% sequence identity with the polypeptide having the amino acid sequence set out in figure 6.

5. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide which is a active portion or derivative of the polypeptide having the amino acid sequence set out in figure 6.

6. An isolated nucleic acid molecule which comprises a fragment of the nucleotide sequence as set out in figure 6 or figure 11 which is greater than 20 nucleotides in length with the proviso that the nucleic acid molecule is not an EST selected from the group consisting of IB3089, R42707, T15661, T15474, AA011030, Z41452, F08986, R40799, H10959, R42933, W50233 or W65061 all available from GeneBank.

7. An isolated nucleic acid molecule comprising the nucleotide sequence of the DBCCR1 promoter region as set out in figure 11.

8. The nucleic acid molecule of claim 7 wherein the DBCCR1 promoter region is operably linked to a reporter

gene.

9. The nucleic acid molecule of claim 7 wherein the DBCCR1 promoter region is operably linked to the nucleic acid sequence of any one of claims 1 to 5.

10. An expression vector comprising the nucleic acid molecule of any one of claims 1 to 9.

11. A host cell transformed with the vector of claim 10.

12. A method of producing DBCCR1 polypeptide comprising culturing host cells transformed with an expression vector comprising the nucleic acid of any one of claims 1 to 5.

13. The method of claim 12 further comprising the step of recovering the DBCCR1 polypeptide.

14. A substance which is an isolated polypeptide comprising a polypeptide having the amino acid sequence set out in figure 6.

15. A substance which is an isolated polypeptide having greater than 80% sequence identity with the amino acid sequence set out in figure 6.

16. A substance which is a polypeptide which is a derivative or allele of the DBCCR1 polypeptide having the sequence set out in figure 6.

17. A substance which is a fragment of the DBCCR1 polypeptide having greater than seven contiguous amino acids having sequence identity with the amino acid sequence set out in figure 6.

18. A substance which is an active portion of a DBCCR1 polypeptide having the amino acid sequence of figure 6.

19. The substance of any one of claims 14 to 18 which is linked to a coupling partner.

20. The substance of claim 19 wherein the coupling partner is an effector molecule, a label, a drug, a toxin, a carrier or a transport molecule.

21. A nucleic acid molecule of any one of claims 1 to 9 for use in a method of medical treatment.

22. Use of a nucleic acid molecule of any one of claims 1 to 5 for the preparation of a medicament for the treatment of cancer.

23. The use of claim 22 wherein the nucleic acid is expressed in a population of cells to produce biologically active DBCCR1 polypeptide.

24. The use of claim 22 wherein a viral vector is used to deliver the nucleic acid to the population of cells.

25. The use of claim 22 or 23 wherein the DBCCR1 polypeptide has the biological activity of inhibiting cell growth.

26. The use of any one of claims 22 to 25 wherein the population of cells are tumour cells.

27. The use of any one of claims 22 to 26 wherein the cancer is bladder cancer, squamous carcinoma, skin cancer, renal cell carcinoma, squamous cell carcinoma of the oesophagus or ovarian cancer.

28. A polypeptide of any one of claims 14 to 20 for use in a method of medical treatment.

29. Use of polypeptide of any one of claims 14 to 20 for

use the preparation of medicament for the treatment of cancer.

30. The use of claim 29 wherein the cancer is bladder cancer, squamous carcinoma, skin cancer, renal cell carcinoma, squamous cell carcinoma of the esophagus and ovarian cancer.

31. An antibody which is capable of specifically binding to a DBCCR1 polypeptide of any one of claims 14 to 20.

32. Use of a polypeptide of any one of claims 14 to 20 in the preparation of an antibody which is capable of specifically binding to the polypeptide.

33. A pharmaceutical composition comprising the nucleic acid molecule of any one of claims 1 to 9 in combination with a pharmaceutically acceptable carrier.

34. A pharmaceutical composition comprising a DBCCR1 polypeptide of any one of claims 14 to 20 in combination with a pharmaceutically acceptable carrier.

35. Use of a methylation inhibitor for the preparation of a medicament for increasing the expression of the DBCCR1 gene having the nucleic acid sequence as set out in figure 6.

36. The use of claim 36 wherein the methylation inhibitor is methylation inhibitor 5-aza-2'-deoxycytidine.

35. A method of determining inactivation of the DBCCR1 gene in a patient, the method comprising:

obtaining from the patient a nucleic acid sample comprising the DBCCR1 promoter region having the sequence set out in figure 11 and/or the DBCCR1 nucleic acid having the sequence set out in figure 6; and,

determining the extent of methylation of the nucleic acid, wherein hypermethylation of the nucleic acid indicates inactivation of the DBCCR1 gene.

36. The method of claim 35 wherein the method comprises the further step of correlating the inactivation of the DBCCR1 gene to the presence of a tumour or a predisposition of the patient to cancer.

37. The method of claim 35 or claim 36 wherein the methylation of the nucleic acid is determined using methylation sensitive single nucleotide primer extension (Ms-SNuPE).

38. The method of claim 35 or claim 36 wherein the methylation of nucleic acid is determined by digestion with methylation sensitive restriction enzymes, followed by Southern analysis.

39. The method of claim 35 or claim 36 wherein the methylation of the nucleic acid is determined by digestion with methylation sensitive restriction enzymes followed by PCR amplification of the nucleic acid.

40. The method of claim 35 or claim 36 wherein the nucleic acid is treated with bisulphite to cause unmethylated cytosine in the nucleic acid sample to be converted to uracil and the bisulphite treated nucleic acid is amplified by PCR.

41. The method of claim 40 wherein the extent of methylation of the amplified nucleic acid is determined by restriction enzyme digestion or by sequecning of the pCR product.

42. The method of any one of claims 35 to 40 wherein the cancer is associated with loss of heterozygosity involving

9q32-33.

43. The method of claim 42 wherein the cancer is bladder cancer, squamous carcinoma, skin cancer, renal cell carcinoma, oesophageal cancer and/or ovarian cancer.

44. A method of determining the extent of inactivation of the DBCCR1 gene, the method employing a biological sample from a patient and comprising:

(a) determining the level of expression of the polypeptide encoded by the DBCCR1 gene and having the amino acid sequence set out in figure 6 in a sample from a patient; and/or;

(b) determining whether the 5' end of the DBCCR1 nucleic acid having the nucleotide sequence set out in figure 6 or the DBCCR1 promoter region having the nucleotide sequence set out in figure 11 are hypermethylated in a sample from patient; and/or,

(c) determining whether all or a part of at least one of the DBCCR1 alleles is deleted in a nucleic acid sample from a patient;

wherein inactivation of the DBCCR1 gene indicates the presence of a tumour or a predisposition of the patient to cancer.

45. The method of claim 44 wherein the determination under (c) is assessed using Southern blotting or quantitative duplex PCR.

46. The method of claim 44 or claim 45 wherein the biological sample is blood, plasma, serum, tissue samples, tumour samples, saliva or urine.

47. A method for detecting mutations in the DBCCR1 gene or the polypeptide encoded by the gene as set out in figure 6, the methods including:

(a) comparing the sequence of nucleic acid in the

sample with the DBCCR1 nucleic acid sequence to determine whether the sample from the patient contains mutations; or,

(b) determining the presence in a sample from a patient of the polypeptide encoded by the DBCCR1 gene and, if present, determining whether the polypeptide is full length, and/or is mutated, and/or is expressed at the normal level; or,

(c) using DNA fingerprinting to compare the restriction pattern produced when a restriction enzyme cuts a sample of nucleic acid from the patient with the restriction pattern obtained from normal DBCCR1 gene or from known mutations thereof; or,

(d) using a specific binding member capable of binding to a DBCCR1 nucleic acid sequence (either a normal sequence or a known mutated sequence), the specific binding member comprising nucleic acid hybridisable with the DBCCR1 sequence, or substances comprising an antibody domain with specificity for a native or mutated DBCCR1 nucleic acid sequence or the polypeptide encoded by it, the specific binding member being labelled so that binding of the specific binding member to its binding partner is detectable; or,

(e) using PCR involving one or more primers based on normal or mutated DBCCR1 gene sequence to screen for normal or mutant DBCCR1 gene in a sample from a patient.

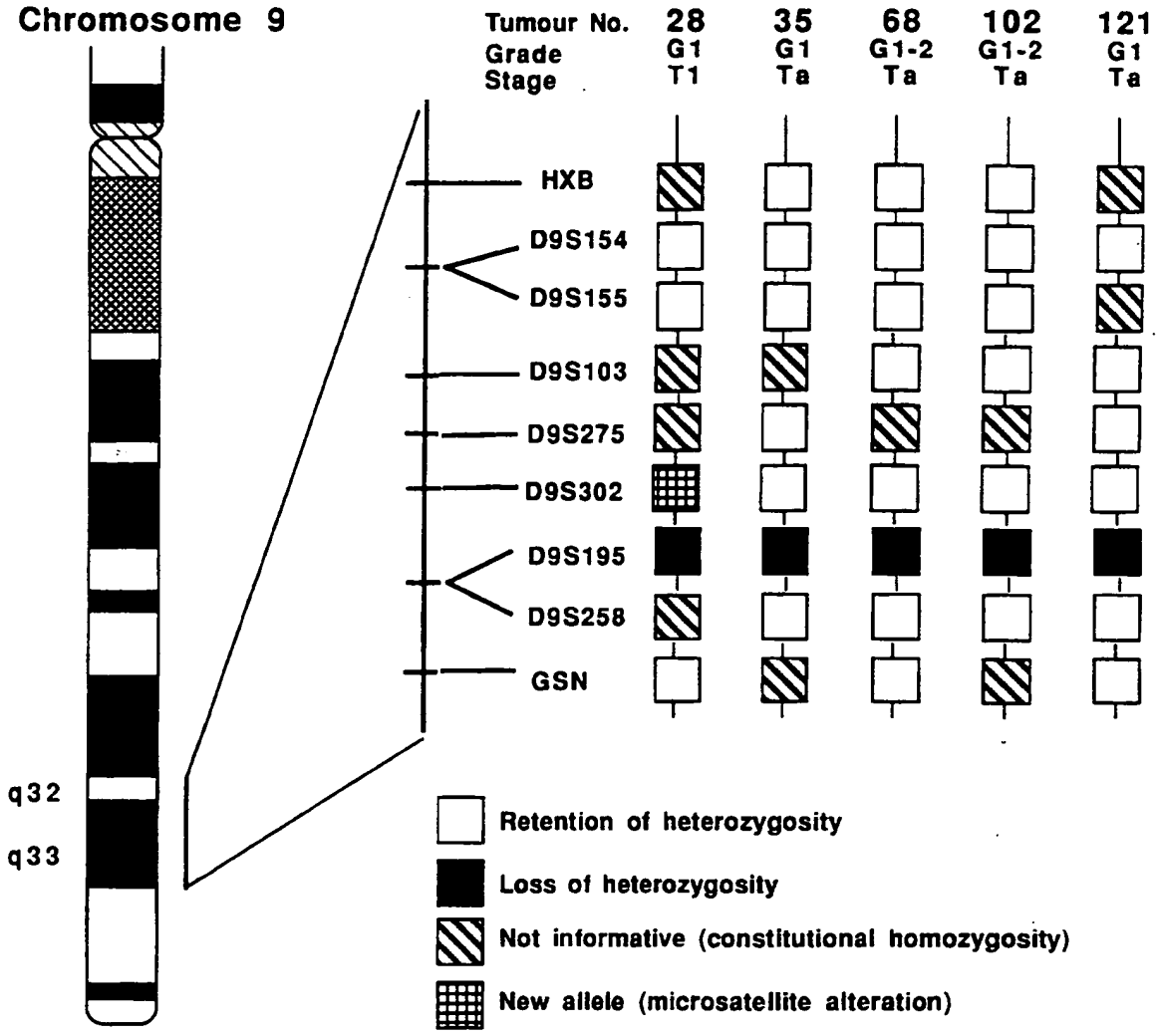
48. A method of screening for compounds capable activating production of DBCCR1 polypeptide, the method employing a nucleic acid construct comprising a functional part of the promoter having the nucleotide sequence as set out in figure 11, the promoter being operably linked to a reporter gene capable of producing a detectable signal, the method comprising exposing the construct to candidate compounds and detecting the signal produced by the reporter gene.

49. Use of the nucleic acid sequence set out in figure 6 or figure 11 in the design of primers for use in the

polymerase chain reaction to amplify all or part of the DBCCR1 nucleic acid sequence.

50. The use of claim 49 employing the primers set out in table 3.

Figure 1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D9S103
D9S275
D9S302
D9S195
D9S258
GSN
q32
q33
Retention of heterozygosity
Loss of heterozygosity
Not informative (constitutional homozygosity)
New allele (microsatellite alteration)

Figure 2

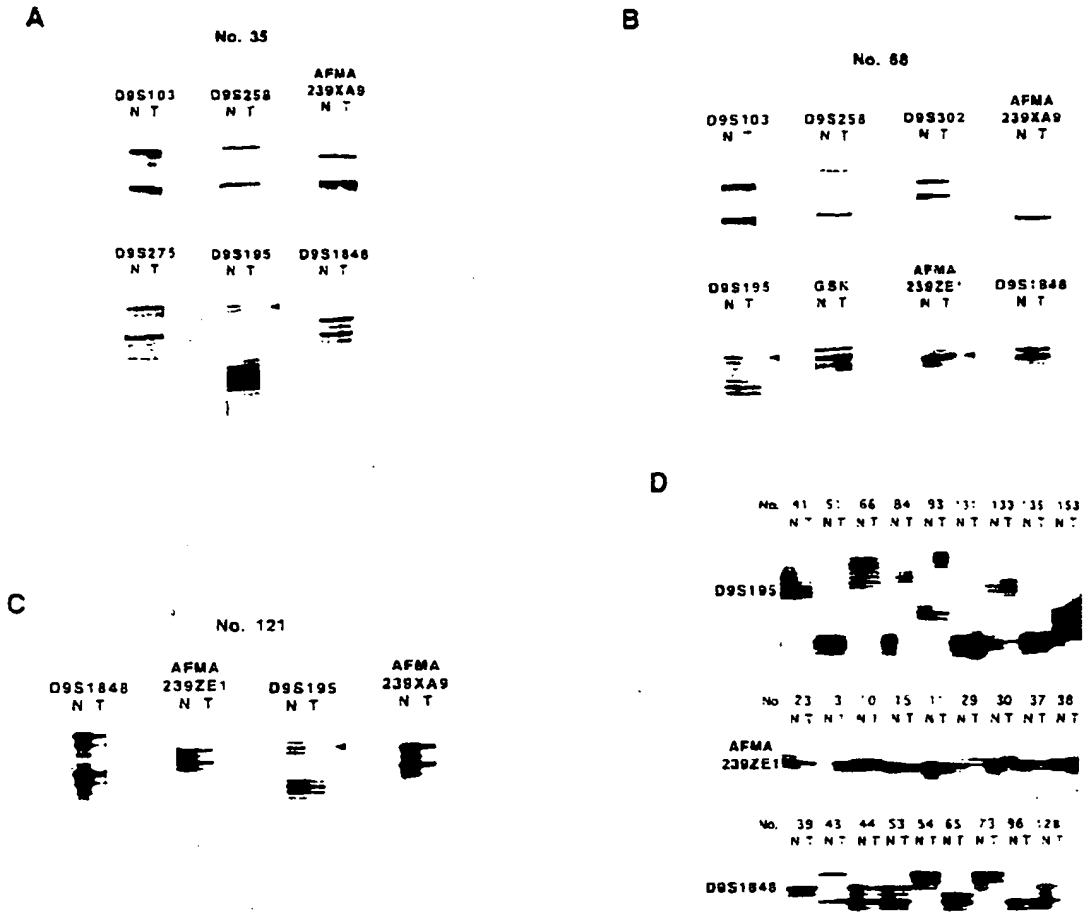
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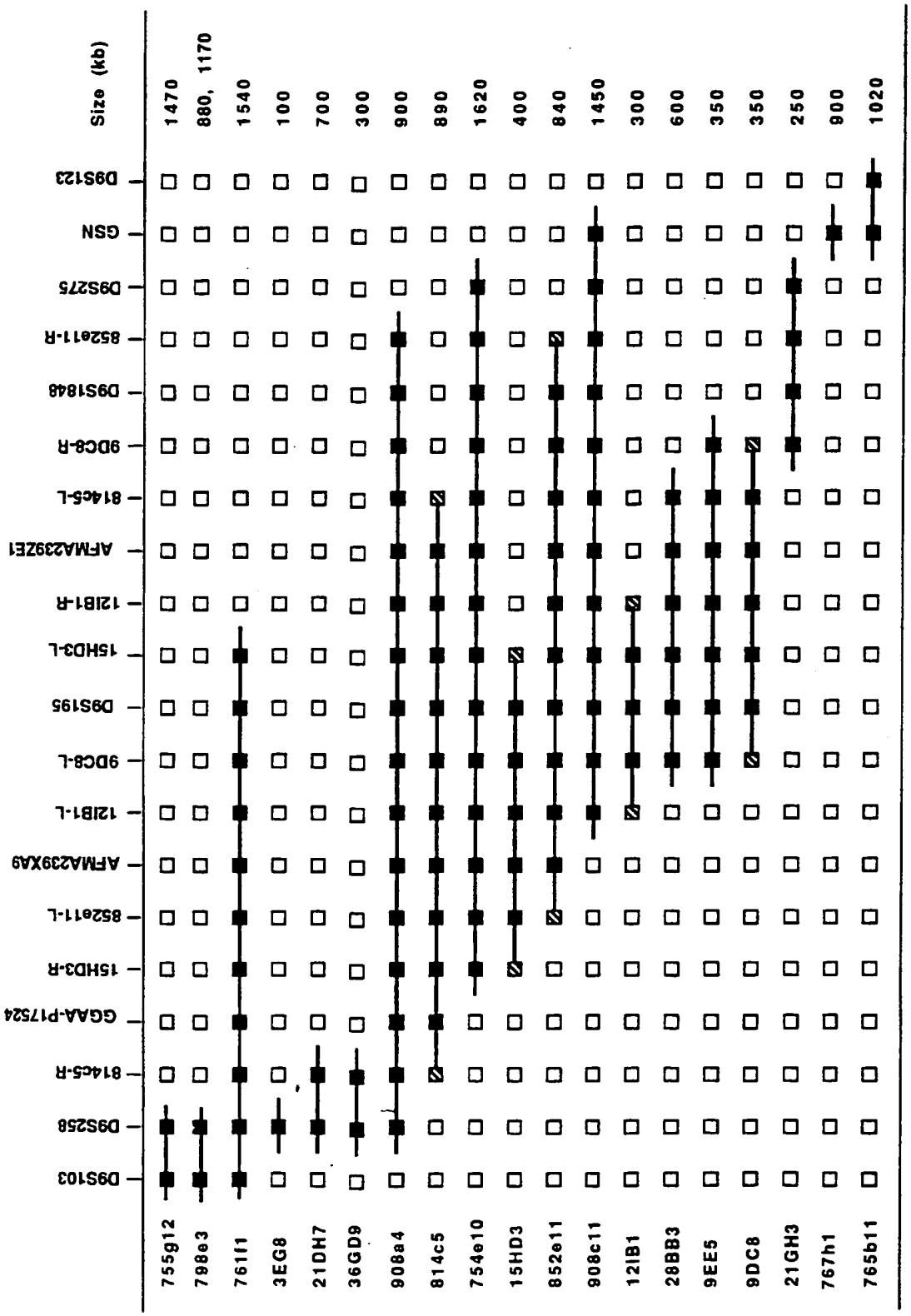
Figure 3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D9S103
755g12
798e3
761I1
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36GD9
908a4
814c5
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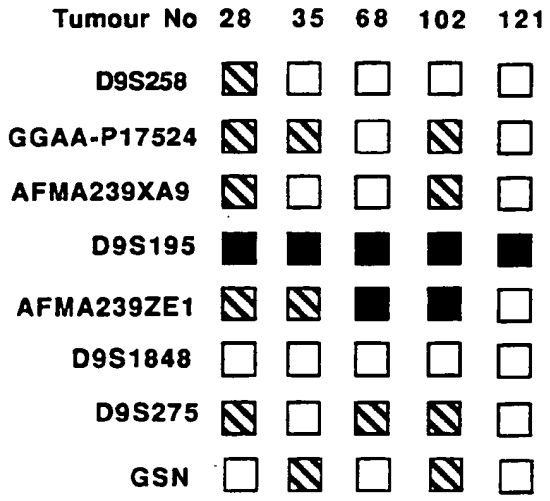
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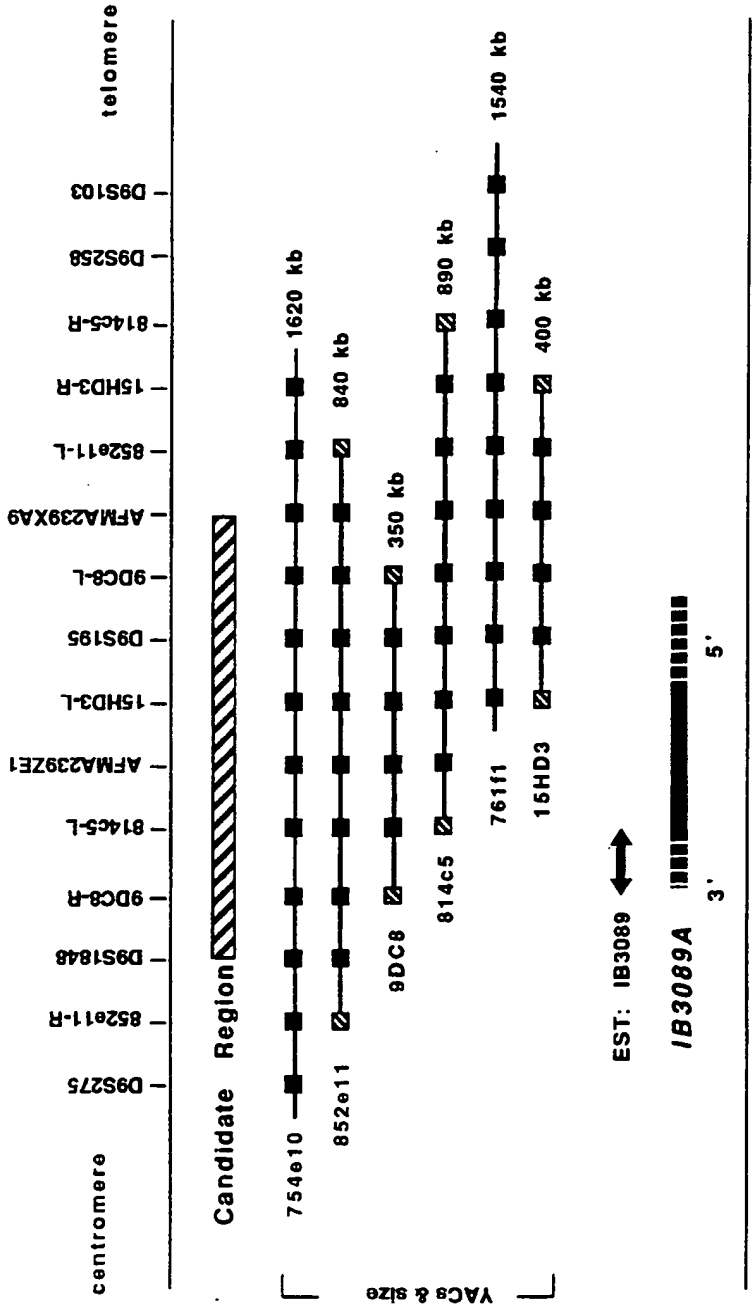
AFMA239XA9
D9S195
AFMA239ZE1
D9S1848
D9S275
GSN

Figure 4

Figure 5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D9S1848
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814c5-L
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AFMA239XA9
852e11-L
15HD3-R
814c5-R
D9S258
D9S103
telomere
Candidate Region
754e10
852e11
9DC8
814c5
761f1
15HD3
1620 kb
840 kb
350 kb
890 kb
1540 kb
400 kb
YACs & size
EST: IB3089
IB3089A
3'
5'

Figure 6


```
1     CCTCGCTCCCGGACCCCACGGCTGCAAACTGATCTGGCGCGCGGGGAGGAGGAGAGCGCA
61    GGCGAGCGAACCCGCGAGAGAGGGAGAGAGCGAGCGAGCAACAGCGAGAGCGAGAGCGAG
121   AGAGCCGGGAGGCAGAGGGAGTAGTGACCGCCTTCCGGAGCCGGGATTCATGCCTGTCCT
181   CGGGACCAGCGAAGGGGACTTTACGGCTGAGTATGAGCCAGGCTGCTAGGAGCCAGGTAC
241   CCCCACGCCTGCAGTCCCCGCGCCGTGCCCGGAATGCGAGCTGGACGCAGGGCTCTCCCA
301   AGTTCCCACCGAGCCGAATAAAAAGCGTCCGCCCGCAGCTCTCCGCCAAAGACGGACATT
            »« exon 2
361   GACTCCAGGACTCCACAGAATGGAGCAAGAATGAGAGGAAAAAGGCCGGCAGAAAAGCAT
                                                                M   1
421   GAACTGGAGGTTTGTTGAGCTCCTCTACTTCCTGTTTATATGGGGCCGTATCTCAGTGCA
       N  W  R  F  V  E  L  L  Y  F  L  F  I  W  G  R  I  S  V  Q   21
481   GCCCTCCCACCAGGAACCAGCTGGGACAGACCAACATGTCTCCAAGGAATTTGATTGGCT
       P  S  H  Q  E  P  A  G  T  D  Q  H  V  S  K  E  F  D  W  L   41
541   CATTTCAGACAGGGGGCCTTTCCACCACTCCAGGAGCTACCTATCCTTTGTGGAAAGACA
       I  S  D  R  G  P  F  H  H  S  R  S  Y  L  S  F  V  E  R  H   61
                                        »« exon 3
601   CCGTCAAGGATTTACAACCAGATATAAAATATACAGGGAGTTTGCCCGTTGGAAGGTGAG
       R  Q  G  F  T  T  R  Y  K  I  Y  R  E  F  A  R  W  K  V  R   81
661   GAACACAGCCATCGAGAGGAGAGATCTGGTCCGCCATCCAGTGCCCCTCATGCCGGAGTT
       N  T  A  I  E  R  R  D  L  V  R  H  P  V  P  L  M  P  E  F  101
721   TCAAAGGAGCATCCGCCTGCTTGGCAGGAGACCTACCACTCAGCAGTTCATCGATACCAT
       Q  R  S  I  R  L  L  G  R  R  P  T  T  Q  Q  F  I  D  T  I  121
                                                   »« exon 4
781   CATCAAAAAGTACGGCACCCACCTGCTCATCTCAGCCACATTGGGAGGGGAGGAGGCTTT
       I  K  K  Y  G  T  H  L  L  I  S  A  T  L  G  G  E  E  A  L  141
841   GACCATGTATATGGACAAAAGTCGCCTCGACAGGAAGTCAGGGAATGCCACTCAAAGTGT
       T  M  Y  M  D  K  S  R  L  D  R  K  S  G  N  A  T  Q  S  V  161
901   TGAAGCTCTGCACCAGCTCGCATCATCCTACTTTGTTGACCGTGATGGTACCATGAGGAG
       E  A  L  H  Q  L  A  S  S  Y  F  V  D  R  D  G  T  M  R  R  181
                                              »« exon 5
961   GCTTCATGAGATCCAGATATCAACTGGAGCAATCAAGGTCACAGAGACACGCACTGGGCC
       L  H  E  I  Q  I  S  T  G  A  I  K  V  T  E  T  R  T  G  P  201
1021  TCTGGGCTGTAACAGTTATGACAATCTGGACTCTGTGAGTTCCGTCCTTCTGCAAAGCAC
       L  G  C  N  S  Y  D  N  L  D  S  V  S  S  V  L  L  Q  S  T  221
                                  »« exon 6
1081  GGAGAGCAAACTGCACCTTCAAGGTCTTCAGATAATCTTTCCTCAGTATCTGCAAGAGAA
       E  S  K  L  H  L  Q  G  L  Q  I  I  F  P  Q  Y  L  Q  E  K  241
1141  GTTTGTCCAGTCGGCCTTGAGCTATATCATGTGCAATGGGGAGGGGGAGTACCTGTGCCA
       F  V  Q  S  A  L  S  Y  I  M  C  N  G  E  G  E  Y  L  C  Q  261
1201  GAACAGCCAGTGTCGCTGCCAATGTGCCGAGGAGTTTCCGCAGTGCAACTGCCCCATCAC
       N  S  Q  C  R  C  Q  C  A  E  E  F  P  Q  C  N  C  P  I  T  281
1261  GGACATCCAGATCATGGAGTACACGCTGGCCAACATGGCCAAGTCTTGGGCCGAAGCTTA
       D  I  Q  I  M  E  Y  T  L  A  N  M  A  K  S  W  A  E  A  Y  301
                              »« exon 7
1321  TAAGGACCTGGAGAATTCAGATGAGTTTAAATCATTTATGAAGCGCCTCCCCAGCAACCA
       K  D  L  E  N  S  D  E  F  K  S  F  M  K  R  L  P  S  N  H  321
1381  CTTCCTGACCATCGGAAGCATCCATCAGCACTGGGGCAATGACTGGGACCTGCAGAACCG
       F  L  T  I  G  S  I  H  Q  H  W  G  N  D  W  D  L  Q  N  R  341
1441  CTACAAGCTCCTGCAGAGTGCCACGGAGGCACAGAGACAAAAGATCCAACGCACTGCCCG
       Y  K  L  L  Q  S  A  T  E  A  Q  R  Q  K  I  Q  R  T  A  R  361
1501  CAAGCTTTTCGGCCTCAGTGTACGCTGTCGCCACAATCCCAACCACCAGCTGCCTAGAGA
       K  L  F  G  L  S  V  R  C  R  H  N  P  N  H  Q  L  P  R  E  381
        »« exon 8
1561  GAGGACAATTCAGCAGTGGCTTGCAAGGGTCCAGTCACTCCTCTACTGTAATGAGAATGG
       R  T  I  Q  Q  W  L  A  R  V  Q  S  L  L  Y  C  N  E  N  G  401
1621  GTTTTGGGGAACCTTCCTGGAGAGCCAGCGGAGCTGCGTGTGCCACGGCAGCACCACGCT
       F  W  G  T  F  L  E  S  Q  R  S  C  V  C  H  G  S  T  T  L  421
1681  GTGCCAGCGCCCCATCCCCTGCGTGATAGGCGGGAACAACAGCTGCACCATGTGCAGCCT
       C  Q  R  P  I  P  C  V  I  G  G  N  N  S  C  T  M  C  S  L  441
```

Figure 6 ... continued

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1741  GGCCAACATCTCCCTCTGTGGCTCCTGCAACAAGGGCTACAAGCTGTATCGAGGCCGCTG
        A  N  I  S  L  C  G  S  C  N  K  G  Y  K  L  Y  R  G  R  C   461
1801  TGAACCACAGAACGTGGACTCGGAGCGGAGCGAGCAGTTCATCAGCTTTGAGACTGACCT
        E  P  Q  N  V  D  S  E  R  S  E  Q  F  I  S  F  E  T  D  L   481
1861  GGACTTCCAGGACCTGGAGCTGAAGTACCTGCTGCAGAAGATGGACTCACGCCTCTACGT
        D  F  Q  D  L  E  L  K  Y  L  L  Q  K  M  D  S  R  L  Y  V   501
1921  CCACACCACCTTCATCAGCAACGAGATCCGCCTCGACACCTTCTTTGACCCTCGGTGGCG
        H  T  T  F  I  S  N  E  I  R  L  D  T  F  F  D  P  R  W  R   521
1981  CAAGCGCATGTCCCTCACTCTCAAGAGCAACAAGAACCGCATGGACTTCATCCACATGGT
        K  R  M  S  L  T  L  K  S  N  K  N  R  M  D  F  I  H  M  V   541
2041  GATCGGCATGTCCATGCGCATCTGCCAGATGCGCAACAGCAGCCTGGACCCCATGTTCTT
        I  G  M  S  M  R  I  C  Q  M  R  N  S  S  L  D  P  M  F  F   561
2101  TGTCTATGTCAACCCCTTTAGCGGGAGCCATTCGGAGGGCTGGAACATGCCCTTCGGGGA
        V  Y  V  N  P  F  S  G  S  H  S  E  G  W  N  M  P  F  G  E   581
2161  ATTTGGCTACCCACGCTGGGAGAAGATCCGTCTCCAAAACAGCCAGTGCTACAACTGGAC
        F  G  Y  P  R  W  E  K  I  R  L  Q  N  S  Q  C  Y  N  W  T   601
2221  TCTTTTGCTGGGCAATCGGTGGAAAACATTTTTCGAGACGGTCCACATCTACCTACGTAG
        L  L  L  G  N  R  W  K  T  F  F  E  T  V  H  I  Y  L  R  S   621
2281  TCGGACTCGGCTACCTACCCTACTGCGAAATGAGACTGGCCAGGGCCCCGTGGACCTGTC
        R  T  R  L  P  T  L  L  R  N  E  T  G  Q  G  P  V  D  L  S   641
2341  GGATCCCTCCAAGAGGCAGTTCTACATCAAGATCTCAGACGTGCAGGTGTTTGGGTATAG
        D  P  S  K  R  Q  F  Y  I  K  I  S  D  V  Q  V  F  G  Y  S   661
2401  CCTGAGGTTCAACGCCGACCTCCTGCGCAGTGCAGTGCAGCAGGTCAACCAGTCCTACAC
        L  R  F  N  A  D  L  L  R  S  A  V  Q  Q  V  N  Q  S  Y  T   681
2461  ACAGGGCGGCCAGTTCTATTCCTCTTCGTCAGTGATGCTCCTCTTGTTGGATATTCGGGA
        Q  G  G  Q  F  Y  S  S  S  S  V  M  L  L  L  L  D  I  R  D   701
2521  CCGAATTAATCGCCTGGCCCCTCCTGTGGCCCCGGGGAAACCCCAGCTGGACTTGTTCTC
        R  I  N  R  L  A  P  P  V  A  P  G  K  P  Q  L  D  L  F  S   721
2581  CTGTATGCTGAAACACCGCCTGAAACTGACCAACAGCGAGATCATCAGGGTGAACCACGC
        C  M  L  K  H  R  L  K  L  T  N  S  E  I  I  R  V  N  H  A   741
2641  CTTGGACCTGTACAACACGGAGATCCTCAAACAGTCGGACCAGATGACAGCCAAACTCTG
        L  D  L  Y  N  T  E  I  L  K  Q  S  D  Q  M  T  A  K  L  C   761
2701  CTAACCCGGGACTCCTTGCCATGGACTTTTCCTGTTGTTGTACACACACAACAGAACAAA
2761  ATGAAGCAAAACAAAACAAAAAAACCCACAAAAATTTGTAAAATGTAATTAATATTCAAA
2821  GAAAAGGAGGAAAATCTTCATTTGTTGGAAATGAAAACGTTCTAGCAACTGTATAAAAGC
2881  GTTGGGCATGTTTGTTATTTCTATACTCACTCTGTCATGAAGAAGGGTCTCAGCCTTTTG
2941  TGGAGCATTGAGGGAGTTGCTTCTTAGGCCTCAGGTGCTGTATTGGGGGAGAAGGGAGAA
3001  AGCATATGCAATGAATTGTAAAGATCTCTGCTGTGCAGGTGCTAAGATTAAACACTAAAA
3061  AGAAAGAGAGATATATGTAATGTACAACTGACACTGCCATTTTTCCTTTTTGGAGGAAAT
3121  GGACATAGATAAAGAAGATATTTCTTCGGTTAAGATTT    (3158)
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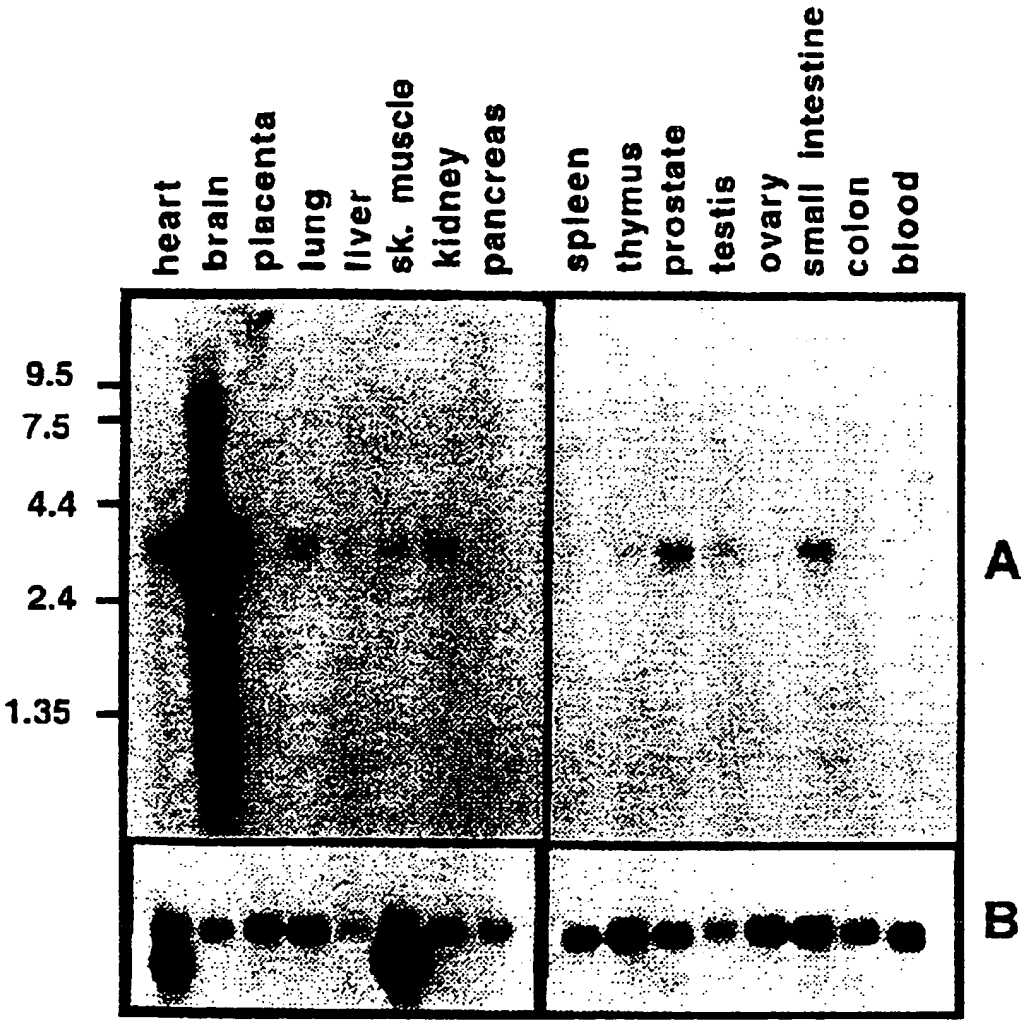
Figure 7


heart
brain
placenta
lung
liver
sk. muscle
kidney
pancreas
spleen
thymus
prostate
testis
ovary
small intestine
colon
blood
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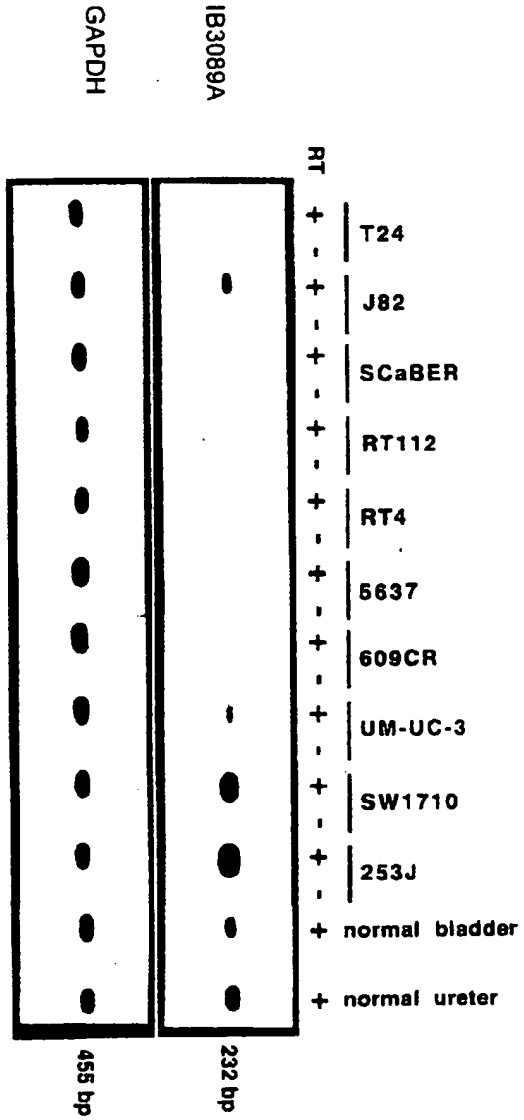
7.5
4.4
2.4
1.35
A
B

Figure 8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IB3089A
RT
+
-
T24
J82
SCaBER
RT112
RT4
5637
609CR
UM-UC-3
SW1710
253J
normal bladder
normal ureter
455 bp
232 bp

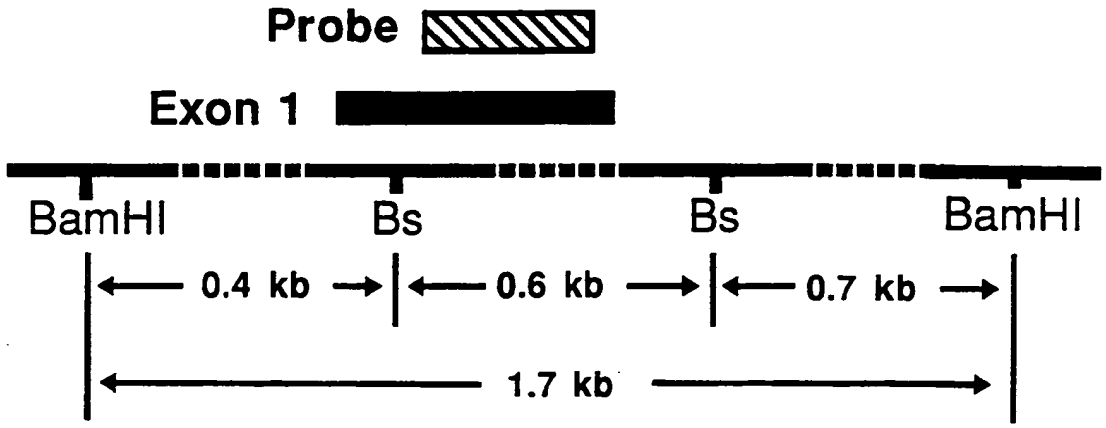
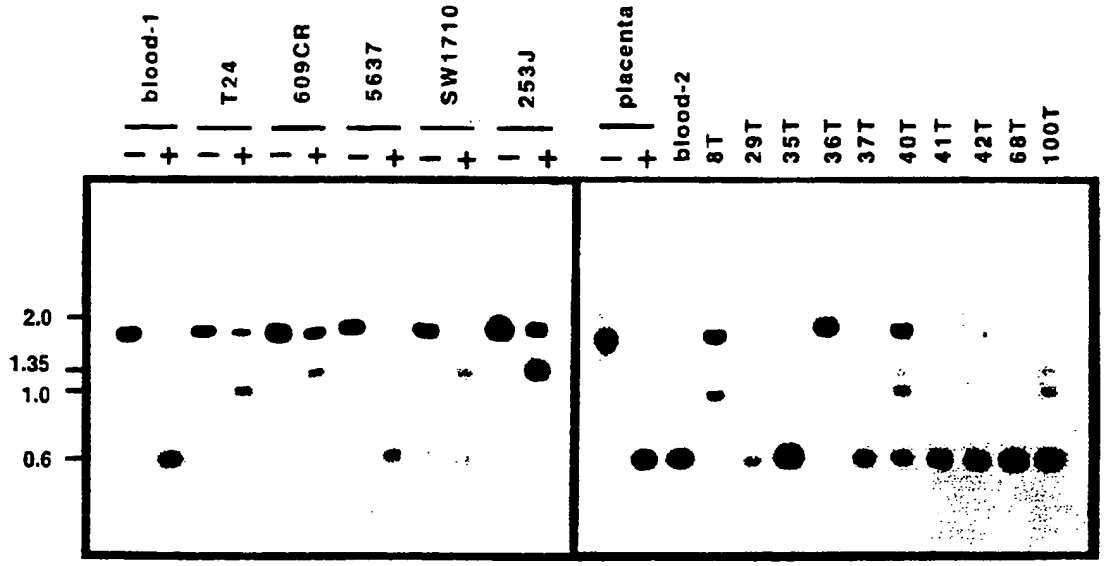


Figure 9a

Figure 9b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42T
68T
100T
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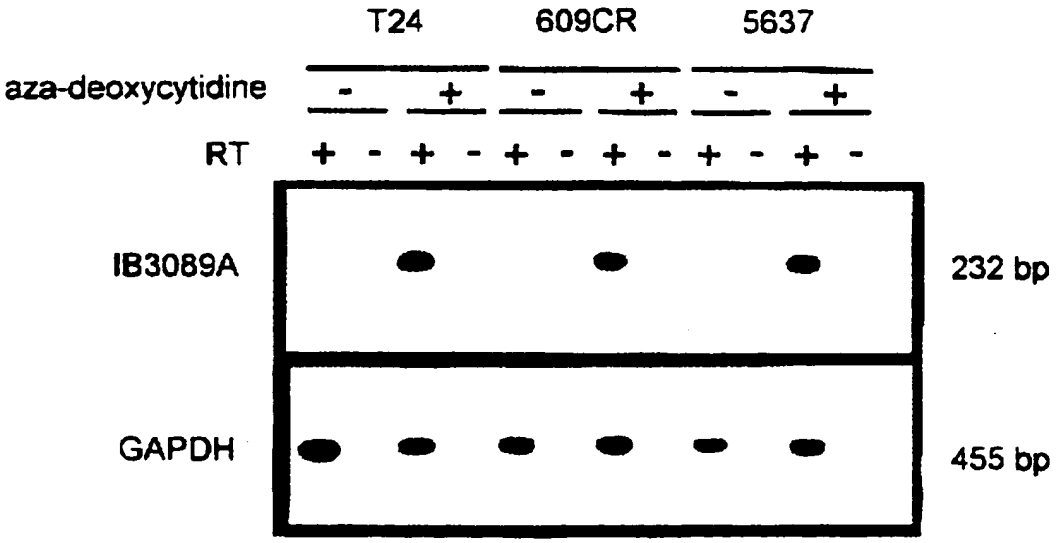
1.35
1.0
0.6

Figure 10


T24
609CR
5637
aza-deoxycytidine
- + - + - +
RT
+ - + - + - + - + - + -
IB3089A
232 bp
GAPDH
455 bp

INTERNATIONAL SEARCH REPORT

Inter .onal Application No
PCT/GB 98/01515

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/74 C12N15/86 C07K16/22 A61K31/70
C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	HABUCHI T. ET AL.: "Structure and methylation-based silencing of a gene (DBCCR1) within a candidate bladder cancer tumor suppressor region at 9q32-q33" GENOMICS, vol. 48, no. 3, 15 March 1998, pages 277-288, XP002080177 see the whole document	1-6, 14-18, 28, 35-38, 40, 46-49, 51,52
X	Database EMBL:Emest14, entry HSZZ24881 Accession number AA319716 18 April 1997 97% identity with Seq.ID:1 nt.342-609 XP002080183	6
A	see the whole document	1

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 October 1998	21/10/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Macchia, G

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INTERNATIONAL SEARCH REPORT

Inte. .onal Application No
PCT/GB 98/01515

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	Database EMBL:Emest14, entry HSZZ31387 Accession number AA326334 18 April 1997 99% identity with Seq.ID:1 nt.679-907 XP002080184	6
A	see the whole document	1
X	Database EMBL:Emest11, entry HSA11152 Accession number AA011152 2 August 1996 98% identity with Seq.ID:1 nt.1045-1304 XP002080185	6
A	see the whole document	1
X	Database EMBL:Emest11, entry HS958161 Accession number H10958 2 July 1995 94% identity with Seq.ID:1 nt.1431-1890 XP002080186	6
A	see the whole document	1
X	Database EMBL:Emsts, entry HS433354 Accession number G23433 1 June 1996 97% identity with Seq.ID:1 nt.2334-2794 reverse orientation XP002080187	6
A	see the whole document	1
X	ZINGG J.-M. AND JONES P.A.: "Genetic and epigenetic aspects of DNA methylation on genome expression, evolution, mutation and carcinogenesis" CARCINOGENESIS, vol. 18, no. 5, May 1997, pages 869-882, XP002080178 cited in the application	35,36
A	see page 872, right-hand column - page 874 see page 876, left-hand column, paragraph 3 - page 877, left-hand column	37,38, 44,45
X	TAYLOR S.M.: "5-Aza-2'-Deoxycytidine: cell differentiation and DNA methylation" LEUKEMIA, vol. 7, no. SUPPL. 01, 1993, pages 3-8, XP002066107 see abstract	35,36

-/--

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INTERNATIONAL SEARCH REPORT

Inte. .onal Application No
PCT/GB 98/01515

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CÔTÉ S. AND MOMPARLER R.L.: "Activation of the retinoic acid receptor beta gene by 5-aza-2'-deoxycytidine in human DLD-1 colon carcinoma cells" ANTI-CANCER DRUGS, vol. 8, no. 1, January 1997, pages 56-61, XP002080179 see abstract	35,36
X	MOMPARLER R.L. ET AL.: "Pharmacological approach for optimization of the dose schedule of 5-Aza-2'-deoxycytidine (Decitabine) for the therapy of leukemia" LEUKEMIA, vol. 11, no. Suppl. 1, March 1997, pages 1-6, XP002080180 see abstract	35,36
A	HABUCHI T. ET AL.: "Detailed deletion mapping of chromosome 9q in bladder cancer: evidence for two tumour suppressor loci" ONCOGENE, vol. 11, no. 8, 19 October 1995, pages 1671-1674, XP002080181 cited in the application see abstract	1
A	WO 96 14877 A (THE JOHNS HOPKINS UNIVERSITY SCHOOL OF MEDICINE; BAYLIN; WALES (US)) 23 May 1996 see page 19, line 10 - page 21, line 20	37,38,46
A	SINGER-SAM J. ET AL.: "A quantitative HpaII-PCR assay to measure methylation of DNA from a small number of cells" NUCLEIC ACIDS RESEARCH, vol. 18, no. 3, 1990, page 687 XP002080182 see the whole document	40,41

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 98/ 01515

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. [X] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Claims Nos.: 6, 10, 11, 21, 33, 37-48, 51, 52 all partially, 7-9, 50 all totally

Remark : Due to lack of Figure 11, said claims are not supported by any description enabling their search.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte. .onal Application No
PCT/GB 98/01515

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9614877 A	23-05-1996	US 5756668 A	26-05-1998
		AU 4407196 A	06-06-1996
		BR 9509674 A	16-09-1997
		CN 1171743 A	28-01-1998
		EP 0792168 A	03-09-1997
		FI 972066 A	14-07-1997
		NO 972228 A	14-07-1997